

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 June 2001 (07.06.2001)

PCT

(10) International Publication Number
WO 01/39802 A1

- (51) International Patent Classification⁷: **A61K 39/12**, 39/193, C12N 7/01
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- (21) International Application Number: PCT/US00/32821
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (22) International Filing Date: 1 December 2000 (01.12.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
09/452,638 1 December 1999 (01.12.1999) US
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
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Published:

— With international search report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/39802 A1

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ATTORNEY DOCKET NUMBER: 11219-027-999

SERIAL NUMBER: 10/679,036

REFERENCE: B30

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
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(54) Title: CHIMERIC FLAVIVIRUS VACCINES

(57) Abstract: A chimeric live, infectious, attenuated virus containing a yellow fever virus, in which the nucleotide sequence for a prM-E protein is either deleted, truncated, or mutated, so that functional prM-E protein is not expressed, and integrated into the genome of the yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that the prM-E protein of the second flavivirus is expressed.

CHIMERIC FLAVIVIRUS VACCINES

5 Background of the Invention

This invention relates to infectious, attenuated viruses useful as vaccines against diseases caused by flaviviruses.

Several members of the flavivirus family pose current or potential threats to global public health. For example, Japanese encephalitis is a significant public health problem involving millions of at risk individuals in the Far East. Dengue virus, with an estimated annual incidence of 100 million cases of primary dengue fever and over 450,000 cases of dengue hemorrhagic fever worldwide, has emerged as the single most important arthropod-transmitted human disease.

15 Other flaviviruses continue to cause endemic diseases of variable nature and have the potential to emerge into new areas as a result of changes in climate, vector populations, and environmental disturbances caused by human activity. These flaviviruses include, for example, St. Louis encephalitis virus, which causes sporadic, but serious, acute disease in the midwest, southeast, and western United States; West Nile virus, which causes febrile illness, occasionally complicated by acute encephalitis, and is widely distributed throughout Africa, the Middle East, the former Soviet Union, and parts of Europe; Murray Valley encephalitis virus, which causes endemic nervous system disease in Australia; and
20 Tick-borne encephalitis virus, which is distributed throughout the former Soviet Union and eastern Europe, where its *Ixodes* tick vector is prevalent and responsible for a serious form of encephalitis in those regions.

Hepatitis C virus (HCV) is another member of the flavivirus family, with a genome organization and replication strategy that are similar, but

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not identical, to those of the flaviviruses mentioned above. HCV is transmitted mostly by parenteral exposure and congenital infection, is associated with chronic hepatitis that can progress to cirrhosis and hepatocellular carcinoma, and is a leading cause of liver disease requiring
5 orthotopic transplantation in the United States.

The Flaviviridae family is distinct from the alphaviruses (*e.g.*, WEE, VEE, EEE, SFV, etc.) and currently contains three genera, the flaviviruses, the pestiviruses, and the hepatitis C viruses. Fully processed mature virions of flaviviruses contain three structural proteins, envelope
10 (E), capsid (C), and membrane (M), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Immature flavivirions found in infected cells contain pre-membrane (prM) protein, which is the precursor to the M protein.

After binding of virions to host cell receptors, the E protein
15 undergoes an irreversible conformational change upon exposure to the acidic pH of endosomes, causing fusion between the envelope bilayers of the virions and endocytic vesicles, thus releasing the viral genome into the host cytosol. PrM-containing tick-borne encephalitis (TBE) viruses are fusion-incompetent, indicating that proteolytic processing of prM is
20 necessary for the generation of fusion-competent and fully infectious virions (Guirakhoo *et al.*, J. Gen. Virol. 72(Pt. 2):333-338, 1991). Using ammonium chloride late in the virus replication cycle, prM-containing Murray Valley encephalitis (MVE) viruses were produced and shown to be fusion incompetent. By using sequence-specific peptides and
25 monoclonal antibodies, it was demonstrated that prM interacts with amino acids 200-327 of the E protein. This interaction is necessary to protect the

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E protein from the irreversible conformational changes caused by maturation in the acidic vesicles of the exocytic pathway (Guirakhoo *et al.*, Virology 191:921-931, 1992).

The cleavage of prM to M protein occurs shortly before release of virions by a furin-like cellular protease (Stadler *et al.*, J. Virol. 71:8475-8481, 1997), which is necessary to activate hemagglutinating activity, fusogenic activity, and infectivity of virions. The M protein is cleaved from its precursor protein (prM) after the consensus sequence R-X-R/K-R (X is variable), and incorporated into the virus lipid envelope together with the E protein.

Cleavage sequences have been conserved not only within flaviviruses, but also within proteins of other, unrelated viruses, such as PE2 of murine coronaviruses, PE2 of alphaviruses, HA of influenza viruses, and p160 of retroviruses. Cleavage of the precursor protein is essential for virus infectivity, but not particle formation. It was shown that, in case of a TBE-dengue 4 chimera, a change in the prM cleavage site resulted in decreased neurovirulence of this chimera (Pletnev *et al.*, J. Virol. 67:4956-4963, 1993), consistent with the previous observation that efficient processing of the prM is necessary for full infectivity (Guirakhoo *et al.*, 1991, *supra*; Guirakhoo *et al.*, 1992, *supra*; Heinz *et al.*, Virology 198:109-117, 1994). Antibodies to prM protein can mediate protective immunity, apparently due to neutralization of released virions that contain some uncleaved prM. The proteolytic cleavage site of the PE2 of VEE (4 amino acids) was deleted by site-directed mutagenesis of the infectious clone (Smith *et al.*, ASTMH meeting, December 7-11, 1997). Deletion mutants replicated with high efficiency and PE2 proteins were incorporated into particles. This mutant was evaluated in lethal mouse and

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hamster models and shown to be attenuated; in non-human primates it caused 100% seroconversion and protected all immunized monkeys from a lethal challenge.

5

Summary of the Invention

The invention features chimeric, live, infectious, attenuated viruses that are each composed of:

- (a) a first yellow fever virus (*e.g.*, strain 17D), representing a live, attenuated vaccine virus, in which the nucleotide sequence encoding the prM-E protein is either deleted, truncated, or mutated so that the functional prM-E protein of the first flavivirus is not expressed, and
- (b) integrated into the genome of the first flavivirus, a nucleotide sequence encoding the viral envelope (prM-E) protein of a second, different flavivirus, so that the prM-E protein of the second flavivirus is expressed from the altered genome of the first flavivirus.

The chimeric virus is thus composed of the genes and gene products responsible for intracellular replication belonging to the first flavivirus and the genes and gene products of the envelope of the second flavivirus. Since the viral envelope contains antigenic determinants responsible for inducing neutralizing antibodies, the result of infection with the chimeric virus is that such antibodies are generated against the second flavivirus.

A preferred live virus for use as the first yellow fever virus in the chimeric viruses of the invention is YF 17D, which has been used for human immunization for over 50 years. YF 17D vaccine is described in a number of publications, including publications by Smithburn *et al.* ("Yellow Fever Vaccination," World Health Org., p. 238, 1956), and Freestone (*in Plotkin et al.*, (Eds.), *Vaccines*, 2nd edition, W.B. Saunders,

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Philadelphia, 1995). In addition, the yellow fever virus has been studied at the genetic level (Rice *et al.*, Science 229:726-733, 1985) and information correlating genotype and phenotype has been established (Marchevsky *et al.*, Am. J. Trop. Med. Hyg. 52:75-80, 1995). Specific examples of yellow fever substrains that can be used in the invention include, for example, YF 17DD (GenBank Accession No. U17066), YF 17D-213 (GenBank Accession No. U17067), YF 17D-204 France (X15067, X15062), and YF-17D-204, 234 US (Rice *et al.*, Science 229:726-733, 1985; Rice *et al.*, New Biologist 1:285-296, 1989; C 03700, K 02749). Yellow Fever virus strains are also described by Galler *et al.*, Vaccine 16 (9/10):1024-28, 1998.

Preferred flaviviruses for use as the second flavivirus in the chimeric viruses of the invention, and thus sources of immunizing antigen, include Japanese Encephalitis (JE, *e.g.*, JE SA14-14-2), Dengue (DEN, *e.g.*, any of Dengue types 1-4; for example, Dengue-2 strain PUO-218) (Gruenberg *et al.*, J. Gen. Virol. 67:1391-1398, 1988) (sequence appendix 1; nucleotide sequence of Dengue-2 insert; Pr-M: nucleotides 1-273; M: nucleotides 274-498; E: nucleotides 499-1983) (sequence appendix 1; amino acid sequence of Dengue-2 insert; Pr-M: amino acids 1-91; M: amino acids 92-166; E: amino acids 167-661), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE) (*i.e.*, Central European Encephalitis (CEE) and Russian Spring-Summer Encephalitis (RSSE) viruses), and Hepatitis C (HCV) viruses. Additional flaviviruses for use as the second flavivirus include Kunjin virus, Powassan virus, Kyasanur Forest Disease virus, and Omsk Hemorrhagic Fever virus. As is discussed further below, the second flavivirus sequences can be provided from two different second flaviviruses, such as two Dengue strains.

It is preferable to use attenuated inserts, for example, in the case of inserts from neurotropic viruses, such as JE, MVE, SLE, CEE, and RSSE. In the case of non-neurotropic viruses, such as dengue viruses, it may be preferable to use unmodified inserts, from unattenuated strains.

- 5 Maintenance of native sequences in such inserts can lead to enhanced immunogenicity of the proteins encoded by the inserts, leading to a more effective vaccine.

In a preferred chimeric virus of the invention, the prM-E protein coding sequence of the second flavivirus is substituted for the prM-E
10 protein coding sequence of the live yellow fever virus. Also, as is described further below, the prM portion of the protein can contain a mutation or mutations that prevent cleavage to generate mature membrane protein. Finally, as is discussed in detail below, the chimeric viruses of the invention include the prM signal of yellow fever virus.

- 15 Also included in the invention are methods of preventing or treating flavivirus infection in a mammal, such as a human, by administering a chimeric flavivirus of the invention to the mammal; use of the chimeric flaviviruses of the invention in the preparation of medicaments for preventing or treating flavivirus infection; nucleic acid molecules
20 encoding the chimeric flaviviruses of the invention; and methods of manufacturing the chimeric flaviviruses of the invention. Further, the invention includes use of the chimeric flaviviruses and of the invention in the preparation of medicaments for administering therapeutic gene products to patients, such as cancer patients.

- 25 The invention provides several advantages. For example, because they are live and replicating, the chimeric viruses of the invention can be used to produce long-lasting protective immunity. Also, because the viruses have the replication genes of an attenuated virus (e.g., Yellow

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Fever 17D), the resulting chimeric virus is attenuated to a degree that renders it safe for use in humans.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

5

Brief Description of the Drawings

Fig. 1A is a schematic representation of processing events at the C/prM junction of parental viruses that can be used in the invention.

Fig. 1B is a schematic representation of the sequences in the capsid, prM signal, and prM regions of flaviviruses that can be used in the invention (SEQ ID NOs:54-70).

Fig. 2 is a schematic representation of the approach to making chimeric flaviviruses at the prM signal region used (SEQ ID NOs:71 and 72) by C.J. Lai (WO 93/06214).

Fig. 3 is a schematic representation of an attempt to use the method of C.J. Lai (WO 93/06214) with a yellow fever backbone (SEQ ID NOs:73 and 74).

Fig. 4 is a schematic representation illustrating that the viability of flavivirus chimeras depends on the choice of signal.

Fig. 5 is a schematic representation of the cloning method used in the present invention, at the prM signal region (SEQ ID NOs:75-77).

Fig. 6 is a schematic representation of the C, prM, E, and NS1 regions and junction sequences of a YF/JE chimera of the invention. The amino acid sequences flanking cleavage sites at the junctions are indicated for JE, YF, and the YF/JE chimera (SEQ ID NOs:78-85).

Fig. 7 is a schematic representation of genetic manipulation steps that were carried out to construct a Yellow-Fever/Japanese Encephalitis (YF/JE) chimeric virus of the invention.

Fig. 8 is a set of growth curves for chimeric YF/JE viruses of the invention in cell cultures of vertebrate and mosquito origin.

Fig. 9 is a growth curve of RMS (Research Master Seed, YF/JE SA14-14-2) in Vero and LLC-MK2 cells.

5 Fig. 10 is a graph showing a growth comparison between RMS (YF/JE SA14-14-2) and YF-Vax in MRC-5 cells.

Fig. 11A is a graph showing the effects of indomethacin (IM) or 2-aminopurine (2-AP) on growth kinetics of YF/JE SA14-14-2 (0.01 MOI) in FRhL cells.

10 Fig. 11B is a graph showing the effects of indomethacin (IM) or 2-aminopurine (2-AP) on growth kinetics of YF/JE SA14-14-2 (0.1 MOI) in FRhL cells.

Fig. 12 is a graph and a table showing the results of a mouse neurovirulence analysis carried out with a YF/JE chimeric virus of the invention.

Fig. 13 is a graph showing the neutralizing antibody response of mice immunized with a YF/JE SA14-14-2 chimeric vaccine of the invention. Three week old mice were immunized, and samples for testing were taken at 6 weeks.

20 Fig. 14A is a graph showing the results of neurovirulence testing of YF-Vax in 4 week old ICR mice by the i.c. route.

Fig. 14B is a graph showing the results of neurovirulence testing of YF/JE SA14-14-2 in 4 week old ICR mice by the i.c. route.

Fig. 15 is a set of graphs showing the results of PRNT analysis of neutralizing antibody titers in mice inoculated s.c. with graded doses of YF/JE vaccine. The results in the top graph are 3 weeks post immunization, and the results in the bottom graph are 8 weeks post immunization.

Fig. 16 is a series of graphs showing the serological responses of mice immunized with a single dose of the live viruses indicated in the figure.

Fig. 17 is a set of graphs showing viremia and GMT of viremia in 3
5 rhesus monkeys inoculated with ChimeriVax or YF-Vax by the i.c. route.

Fig. 18 is a graph showing the PRNT neutralizing antibody titers (50%) in rhesus monkeys 2 and 4 weeks post inoculation with a single dose of YF-Vax or ChimeriVax vaccines by the i.c. route.

Fig. 19 is a graph showing the results of neurovirulence testing of
10 YF/JE SA14-14-2 (E-138 K---> mutant).

Fig. 20 is a schematic representation of a two plasmid system for generating chimeric YF/DEN-2 virus. The strategy is essentially as described for the YF/JE chimeric virus.

Fig. 21 is a schematic representation of the structure of modified
15 YF clones designed to delete portions of the NS1 protein and/or express foreign proteins under control of an internal ribosome entry site (IRES). The figure shows only the E/NS1 region of the viral genome. A translational stop codon is introduced at the carboxyl terminus of the envelope (E) protein. Downstream translation is initiated within an
20 intergenic open reading frame (ORF) by IRES-1, driving expression of foreign proteins (*e.g.*, HCV proteins E1 and/or E2). The second IRES (IRES-2) controls translational initiation of the YF nonstructural region, in which nested, truncated NS1 proteins (*e.g.*, NS1del-1, NS1del-2, or NS1del-3) are expressed. The size of the NS1 deletion is inversely
25 proportional to that of the ORF linked to IRES-1.

Fig. 22 is a graph showing the neurovirulence phenotype of ChimeriVax-Den2 in outbred (CD-1) suckling mice inoculated by the I.C. route with 10,000 PFU/0.02 ml.

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Fig. 23 is a graph showing the neurovirulence phenotype of 17D vaccine (YF-Vax) in outbred (CD-1) suckling mice inoculated by the I.P. route with 1000 PFU/0.02 ml.

Figs. 24 A-C are graphs showing the growth of JE SA14, JE
5 SA14-14-2, ChimeriVax™-JE and YF 17D intrathoracically inoculated mosquitoes: (A) *Cx. tritaeniorhynchus* mosquitoes, (B) *Ae. albopictus* mosquitoes, and (C) *Ae. aegypti*. Mean titer = geometric mean of the titers of three individual mosquitoes; log₁₀ pfu/mosquito.

Figs. 25 A-C are graphs showing the growth of JE SA14, JE
10 SA14-14-2, ChimeriVax™-JE and YF 17D IT orally exposed mosquitoes: (A) *Cx. tritaeniorhynchus* mosquitoes, (B) *Ae. albopictus* mosquitoes, and (C) *Ae. aegypti* mosquitoes. Mean titer = geometric mean of the titers of three individual mosquitoes; log₁₀ pfu/mosquito.

Figs. 26 A and B are graphs showing the growth of virus in IT
15 inoculated *Ae. aegypti* (A) and *Ae. albopictus* (B) mosquitoes.

Fig. 27 is a schematic representation of an overview of construction of a YF/DEN1 chimera of the invention.

Fig. 28 is a schematic representation of a plasmid and fragment map relating to construction of a YF/DEN1 chimera of the invention.

20 Fig. 29 is a schematic representation of RT-PCR amplification of the prM-E region of the PaH881/88 DEN3 virus genome. The virus genome is shown on the top diagram. Regions encoding hydrophobic signals for corresponding downstream proteins are shadowed. The prM-E region was amplified in two fragments (black solid lines). Restriction sites
25 introduced for subsequent in-frame *in vitro* ligation into YF backbone (BstBI and NarI) and cloning (NheI) are indicated.

Fig. 30 is a schematic representation of construction of a YF/DEN3 chimera of the invention. YF- and DEN3-specific sequences are shown as

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shadowed and black boxes, respectively. The chimeric YF/DEN3 genome was reconstituted by *in vitro* ligation of three fragments: the large BstBI-AatII portion of 5'3'/Den3/DXho plasmid, a PCR fragment containing the DEN3-specific part of 5.2/Den3 without the one nucleotide deletion (D1) digested with BstBI and EheI (an isoschizomer of NarI), and the large EheI-AatII fragment of YFM5.2 JE SA14-14-2. Ligation products were linearized with XhoI and then transcribed *in vitro* with SP6 RNA polymerase. Vero PM cells were transfected with *in vitro* RNA transcripts to recover the chimeric virus.

Fig. 31 is a schematic representation of an overview of construction of a YF/DEN4 chimera of the invention.

Fig. 32 is a schematic representation of a plasmid and fragment map relating to construction of a YF/DEN4 chimera of the invention.

Detailed Description

The invention provides chimeric flaviviruses that can be used in vaccination methods against flavivirus infection. Construction and analysis of chimeric flaviviruses of the invention, such as chimeras of yellow fever virus and Japanese Encephalitis (JE), Dengue types 1-4 (DEN 1-4), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE), and Hepatitis C (HCV) viruses are described as follows.

Yellow fever (YF) virus is a member of the *Flaviviridae* family of small enveloped positive-strand RNA viruses. Flavivirus proteins are produced by translation of a single long open reading frame to generate a polyprotein, and a complex series of post-translational proteolytic cleavages of the polyprotein by a combination of host and viral proteases, to generate mature viral proteins (Amberg *et al.*, J. Virol. 73:8083-8094,

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1999; Fields, "Flaviviridae," In *Virology*, Fields (ed.), Raven-Lippincott, New York, 1995, Volume I, p. 937). The virus structural proteins are arranged in the order C-prM-E, where "C" is capsid, "prM" is a precursor of the viral envelope-bound M protein, and "E" is the envelope protein.

- 5 These proteins are present in the N-terminal region of the polyprotein, while the non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are located in the C-terminal region of the polyprotein. The amino termini of prM, E, NS1, and NS4B are generated by host signalase cleavage within the lumen of the endoplasmic reticulum (ER), while most
- 10 cleavages within the non-structural region are mediated by a viral protease complex known as NS2B-NS3 (Fields, "Flaviviridae," In *Virology*, Fields (ed.), Raven-Lippincott, New York, 1995, Volume I, p. 937). In addition, the NS2B-NS3 protease complex is responsible for mediating cleavages at the C terminus of both the C protein and the NS4A protein ((Amberg *et*
- 15 *al.*, J. Virol. 73:8083-8094, 1999).

Several research efforts suggest a regulatory role for NS2B-NS3-mediated cleavage at the C terminus of the capsid protein. This site is the only site in the structural region of polyprotein that is cleaved by the NS2B-NS3 protease and, in addition, it includes a highly

20 conserved dibasic-site motif of flaviviruses, which indicates a functional role (Amberg *et al.*, J. Virol. 68:3794-3802, 1994; Yamshchikov *et al.*, J. Virol. 68:5765-5771, 1994). *In vitro* data from various flavivirus models suggest that efficient generation of the prM protein, an early step in proper release of structural proteins, is dependent on the function of the viral

25 protease at the capsid protein site (see summary in Amberg *et al.*, J. Virol. 73:8083-8094, 1999).

Maintenance of this mechanism of coordinate cleavages by NS2B-NS3 at the C terminus of the capsid protein and signalase at the N

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terminus of prM in the chimeras described below is central to the present invention. In particular, in the chimeras of the present invention, the length of the so-called "prM signal," which separates the two cleavage sites by 20 amino acids in YF (Figs. 1A and 1B), is substantially
5 maintained, to ensure polyprotein proteolytic processing and subsequent growth of chimeric viruses that are created in a YF backbone. A hydrophobic domain within this signal serves to direct the translocation of prM into the ER lumen, where efficient signalase cleavage occurs only after cleavage at the NS2B-NS3 site in the capsid protein (Amberg *et al.*,
10 J. Virol. 73:8083-8094, 1999; Figs. 1A and 1B).

In the chimeras of the present invention, only the regions encoding the membrane and envelope proteins (*i.e.*, the prME region) of a non-yellow fever flavivirus are used to replace the corresponding genes in a yellow fever virus clone. The prM signal of the yellow fever virus
15 backbone is maintained. Another method, described in a patent application by C.J. Lai, WO 93/06214, suggests a universal approach to constructing chimeric flaviviruses, involving cloning the prME region of a donor virus into the backbone of an acceptor virus, such that the prM signal sequence is contributed by the incoming prM protein gene. This
20 approach was illustrated using dengue 4 virus as the backbone (acceptor) and tick-borne encephalitis as the donor prME gene. As is illustrated in Fig. 2, the approach described in WO 93/06214 suggests that variability in this cloning strategy, with other chimeric models using flaviviruses as backbone, will have no effect on proper processing of the resulting
25 polyprotein. That is, that flavivirus prM signals are exchangeable when producing viable chimeric viruses. However, attempts to use this approach with YF as a backbone for the insertion of prME genes of dengue 2 virus to create a chimera in which dengue 2 sequences were

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inserted at the 5' NS2B/NS3 cleavage site (*e.g.*, by R. Galler, see Fig. 3) failed to produce a viable virus, and the use of the 14 amino acid signal of the dengue 2 virus prM in this construct explains why. When the cloning strategy was altered to maintain the YF prM signal of 20 amino acids (*i.e.*,
5 only the Den 2 prME region was replaced), it was successful. The failure to make a viable chimera in YF using the shorter signal of dengue prM demonstrates that the approach described in WO 93/06214 does not work for all chimeras, such as those including a YF backbone.

The explanation of the success of the approach described in WO
10 93/06214, using a dengue virus backbone, is that both the viral protease and the prM signal of dengue viruses were maintained. The dengue prM signal is 6-8 amino acids shorter than that of other flaviviruses, such as YF, TBE, MVE, and JE. Dengue, and chimeric flaviviruses with a dengue backbone, rely on dengue NS2B-NS3 protease complex for eventual
15 signalase cleavage at the prM signal. Possibly dengue strain evolution favors a short signal sequence for optimum cascade-event processing of structural viral proteins, proper assembly, and virus production. If a longer signal is cloned in a dengue chimera, the amino acid additions favors translocation of prME, and perhaps cleavage, but not necessarily
20 optimal viral growth. On the other hand, YF, TBE, MVE, and JE have evolved using a long prM signal, and cloning of a shorter signal in any of these backbones obliterates C-prM-E processing and viral growth (Fig. 4).

Thus, central to the present invention is the length of the prM signal (Fig. 5). The cloning of the prME region of any flavivirus into a YF
25 backbone according to the invention always takes place after the prM signal sequence (*i.e.*, LMTGG/VTL for yellow fever); in this way, all prME chimeras encode the yellow fever prM signal, thus ensuring proper processing of the polyprotein. In addition, it is preferable to maintain the

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length and sequence of the YF prM signal in the chimeras of the invention. That is, preferably, the length of the prM signal is 20 amino acids. Less preferably, the length of the prM signal is 15, 16, 17, 18, 19, or more than 20 amino acids in length. Also, it is preferable that the amino acid

5 sequence of the YF prM signal is maintained in the chimeras of the invention, although this sequence can be modified using, for example, conservative amino acid substitutions. Preferably, the sequence of the prM signal is 100%, less preferably, 90%, 80%, 70%, 60%, 50%, or 40% identical to the YF prM signal.

10 As an example of construction of a chimera of the invention, Fig. 6 illustrates a YF/JE chimera in which the YF NS2B-NS3 protease recognition site is maintained. Thus, the recognition site for cleavage of the cytosolic from membrane-associated portions of capsid is homologous for the YF NS2B-NS3 enzyme. At the C/pr-M junction, the portion of the
15 signalase recognition site upstream of the cleavage site is that of the backbone, YF, and the portion downstream of the cleavage site is that of the insert, JE. At the E/NS1 junction, the portion of the signalase recognition site upstream of the cleavage site is similar to that of the insert, JE (four of five of the amino acids are identical to those of the JE
20 sequence), and the portion downstream of the cleavage site is that of the backbone, YF. It is preferable to maintain this or a higher level of amino acid sequence identity to the viruses that form the chimera. Alternatively, at least 25, 50, or 75% sequence identity can be maintained in the three to five amino acid positions flanking the signalase and NS2B-NS3 protease
25 recognition sites.

Also possible, though less preferable, is the use of any of numerous known signal sequences to link the C and pre-M or E and NS1 proteins of the chimeras (see, *e.g.*, von Heijne, Eur. J. Biochem. 133:17-21, 1983; von

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Heijne, J. Mol. Biol. 184:99-105, 1985) or, for example, using the known sequences for guidance, one skilled in the art can design additional signal sequences that can be used in the chimeras of the invention. Typically, for example, the signal sequence will include as its last residue an amino acid
5 with a small, uncharged side chain, such as alanine, glycine, serine, cysteine, threonine, or glutamine. Other requirements of signal sequences are known in the art (see, *e.g.*, von Heijne, 1983, *supra*; von Heijne, 1985, *supra*).

Following the approach described above, we have succeeded in
10 making viable YF chimeric constructs for JE (section 1) and dengue serotypes 1 through 4 (sections 2-5, respectively). Construction and characterization of these, as well as other, constructs is described further below.

15 *1.0 Construction of cDNA Templates for Generation of YF/JE Chimeric Virus*

The derivation of full-length cDNA templates for YF/JE chimeras of the invention described below employed a strategy similar to that earlier workers used to regenerate YF 17D from cDNA for molecular genetic
20 analysis of YF replication. The strategy is described, *e.g.*, by Nestorowicz *et al.* (Virology 199:114-123, 1994).

Briefly, derivation of a YF/JE chimera of the invention involves the following. YF genomic sequences are propagated in two plasmids (YF5'3'IV and YFM5.2), which encode the YF sequences from nucleotides
25 1-2,276 and 8,279-10,861 (YF5'3'IV) and from 1,373-8,704 (YFM5.2) (Rice *et al.*, The New Biologist 1:285-296, 1989). Full-length cDNA templates are generated by ligation of appropriate restriction fragments derived from these plasmids. This method has been the most reliable for

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ensuring stable expression of YF sequences and generation of RNA transcripts of high specific infectivity.

Our strategy for construction of chimeras involves replacement of YF sequences within the YF5'3'IV and YFM5.2 plasmids by the

5 corresponding JE sequences from the start of the prM protein (nucleotide 478, amino acid 128) through the E/NS1 cleavage site (nucleotide 2,452, amino acid 817). In addition to cloning of JE cDNA, several steps were required to introduce or eliminate restriction sites in both the YF and JE sequences to permit *in vitro* ligation. The structure of the template for

10 regenerating chimeric YF (C)/JE (prM-E) virus is shown in Fig. 7. A second chimera, encoding the entire JE structural region (C-prM-E) was engineered using a similar strategy. The second chimera was not able to generate RNA of high infectivity.

15 1.1 Molecular Cloning of the JE Virus Structural Region

Clones of authentic JE structural protein genes were generated from the JE SA14-14-2 strain (JE live, attenuated vaccine strain), because the biological properties and molecular characterization of this strain are well-documented (see, *e.g.*, Eckels *et al.*, Vaccine 6:513-518, 1988; JE SA14-

20 14-2 virus is available from the Centers for Disease Control, Fort Collins, Colorado and the Yale Arbovirus Research Unit, Yale University, New Haven, Connecticut, which are World Health Organization-designated Reference Centers for Arboviruses in the United States). JE SA14-14-2 virus at passage level PDK-5 was obtained and passaged in LLC-MK₂

25 cells to obtain sufficient amounts of virus for cDNA cloning. The strategy used involved cloning the structural region in two pieces that overlap at an *NheI* site (JE nucleotide 1,125), which can then be used for *in vitro* ligation.

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RNA was extracted from monolayers of infected LLC-MK₂ cells and first strand synthesis of negative sense cDNA was carried out using reverse transcriptase with a negative sense primer (JE nucleotide sequence 2,456-71) containing nested *Xba*I and *Nar*I restriction sites for cloning initially into pBluescript II KS(+), and subsequently into YFM5.2(*Nar*I), respectively. First strand cDNA synthesis was followed by PCR amplification of the JE sequence from nucleotides 1,108-2,471 using the same negative sense primer and a positive sense primer (JE nucleotides sequence 1,108-1,130) containing nested *Xba*I and *Nsi*I restriction sites for cloning into pBluescript and YFM5.2(*Nar*I), respectively. JE sequences were verified by restriction enzyme digestion and nucleotide sequencing. The JE nucleotide sequence from nucleotides 1 to 1,130 was derived by PCR amplification of negative strand JE cDNA using a negative sense primer corresponding to JE nucleotides 1,116 to 1,130 and a positive sense primer corresponding to JE nucleotides 1 to 18, both containing an *Eco*RI restriction site. PCR fragments were cloned into pBluescript and JE sequences were verified by nucleotide sequencing. Together, this represents cloning of the JE sequence from nucleotides 1-2,471 (amino acids 1-792).

20

1.2 Construction of YF5'3'IV/JE and YFM5.2/JE Derivatives

To insert the C terminus of the JE envelope protein at the YF E/NS1 cleavage site, a unique *Nar*I restriction site was introduced into the YFM5.2 plasmid by oligonucleotide-directed mutagenesis of the signalase sequence at the E/NS1 cleavage site (YF nucleotides 2,447-2,452, amino acids 816-817) to create YFM5.2(*Nar*I). Transcripts derived from templates incorporating this change were checked for infectivity and yielded a specific infectivity similar to the parental templates

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(approximately 100 plaque-forming units/250 nanograms of transcript). The JE sequence from nucleotides 1,108 to 2,471 was subcloned from several independent PCR-derived clones of pBluescript/JE into YFM5.2(*NarI*) using the unique *NsiI* and *NarI* restriction sites.

- 5 YF5'3'IV/JE clones containing the YF 5' untranslated region (nucleotides 1-118) adjacent to the JE prM-E region were derived by PCR amplification.

To derive sequences containing the junction of the YF capsid and JE prM, a negative sense chimeric primer spanning this region was used
10 with a positive sense primer corresponding to YF5'3'IV nucleotides 6,625-6,639 to generate PCR fragments that were then used as negative sense PCR primers in conjunction with a positive sense primer complementary to the pBluescript vector sequence upstream of the *EcoRI* site, to amplify the JE sequence (encoded in reverse orientation in the pBluescript vector)
15 from nucleotide 477 (N-terminus of the prM protein) through the *NheI* site at nucleotide 1,125. The resulting PCR fragments were inserted into the YF5'3'IV plasmid using the *NotI* and *EcoRI* restriction sites. This construct contains the SP6 promoter preceding the YF 5'-untranslated region, followed by the sequence: YF (C) JE (prM-E), and contains the
20 *NheI* site (JE nucleotide 1,125) required for *in vitro* ligation.

1.3 Engineering YFM5.2 and YF5'3'IV to Contain Restriction Sites for in vitro Ligation

To use the *NheI* site within the JE envelope sequence as a 5' *in vitro*
25 ligation site, a redundant *NheI* site in the YFM5.2 plasmid (nucleotide 5,459) was eliminated. This was accomplished by silent mutation of the YF sequence at nucleotide 5,461 (T→C; alanine, amino acid 1820). This site was incorporated into YFM5.2 by ligation of appropriate restriction

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fragments and introduced into YFM5.2(*NarI*)/JE by exchange of an *NsiI/NarI* fragment encoding the chimeric YF/JE sequence.

To create a unique 3' restriction site for *in vitro* ligation, a *BspEI* site was engineered downstream of the *AatII* site normally used to generate full-length templates from YF5'3'IV and YFM5.2. (Multiple *AatII* sites are present in the JE structural sequence, precluding use of this site for *in vitro* ligation.) The *BspEI* site was created by silent mutation of YF nucleotide 8,581 (A→C; serine, amino acid 2,860), and was introduced into YFM5.2 by exchange of appropriate restriction fragments. The unique site was incorporated into YFM5.2/JE by exchange of the *XbaI/SphI* fragment, and into the YF5'3'IV/JE(prM-E) plasmids by three-piece ligation of appropriate restriction fragments from these parent plasmids and from a derivative of YFM5.2 (*BspEI*) deleting the YF sequence between the *EcoRI* sites at nucleotides 1 and 6,912.

15

1.4 Exchange of JE Nakayama cDNA into YF/JE Chimeric Plasmids

Because of uncertainty about the capacity of the PCR-derived JE SA14-14-2 structural region to function properly in the context of the chimeric virus, we used cDNA from a clone of the JE Nakayama strain that has been extensively characterized in expression experiments and for its capacity to induce protective immunity (see, *e.g.*, McIda *et al.*, Virology 158:348-360, 1987; the JE Nakayama strain is available from the Centers for Disease Control, Fort Collins, Colorado, and the Yale Arbovirus Research Unit, Yale University, New Haven, Connecticut). The Nakayama cDNA was inserted into the YF/JE chimeric plasmids using available restriction sites (*HindIII* to *PvuII* and *BpmI* to *MunI*) to replace the entire prM-E region in the two plasmid system except for a single amino acid, serine, at position 49, which was left intact in order to

25

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utilize the *NheI* site for *in vitro* ligation. The entire JE region in the Nakayama clone was sequenced to verify that the replaced cDNA was authentic (Table 1).

5 *1.5 Generation of Full-Length cDNA Templates, RNA Transfection, and Recovery of Infectious Virus*

Procedures for generating full-length cDNA templates are essentially as described in Rice *et al.* (The New Biologist 1:285-96, 1989; also see Fig. 7). In the case of chimeric templates, the plasmids

10 YF5'3'IV/JE(prM-E) and YFM5.2/JE are digested with *NheI/BspEI* and *in vitro* ligation is performed using 300 nanograms of purified fragments in the presence of T4 DNA ligase. The ligation products are linearized with *XhoI* to allow run-off transcription. SP6 transcripts are synthesized using 50 nanograms of purified template, quantitated by incorporation of ³H-

15 UTP, and integrity of the RNA is verified by non-denaturing agarose gel electrophoresis. Yields range from 5 to 10 micrograms of RNA per reaction using this procedure, most of which is present as full-length transcripts. Transfection of RNA transcripts in the presence of cationic liposomes is carried out as described by Rice *et al. (supra)* for YF 17D. In

20 initial experiments, LLC-MK₂ cells were used for transfection and quantitation of virus, since we have determined the permissiveness for replication and plaque formation of the parental strains of YF and JE. Table 2 illustrates typical results of transfection experiments using Lipofectin (GIBCO/BRL) as a transfection vehicle. Vero cell lines have

25 also been used routinely for preparation of infectious virus stocks, characterization of labeled proteins, and neutralization tests.

Amplification products from Vero cells were sent to the FDA (CBER) for preparation of the RMS in diploid, Fetal Rhesus lung cells. Fetal rhesus lung cells were received from the ATCC as cultured cells and

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were infected with YF/JE SA14-14-2 (clone A-1) at an MOI of 1.0. After 1 hour of incubation at 37°C, the inoculum was aspirated and replaced with 50 ml of EMEM, containing 2% FBS. Virus was harvested 78 hours later, aliquoted into 1 ml vials (a total of 200 vials) and frozen at -70°C.

- 5 Virus titers were determined in Vero, LLC MK2, and CV-1 cells using a standard plaque assay. Titers (pfu/ml) were 1.6×10^6 in Vero cells, 1.25×10^6 in LLC MK2 cells, and 1.35×10^5 in CV-1 cells.

1.6 Nucleotide Sequencing of Chimeric cDNA Templates

- 10 Plasmids containing the chimeric YF/JE cDNA were subjected to sequence analysis of the JE portion of the clones to identify the correct sequences of the SA14-14-2 and Nakayama envelope protein. The nucleotide sequence differences between these constructs in comparison to the reported sequences (McAda *et al.*, *supra*) are shown in Table 1.

- 15 Five amino acid differences at positions 107, 138, 176, 264, and 279 separate the virulent from the attenuated strains of JE virus. Amino acid differences map to three subregions of Domains I and II of the flavivirus E protein model (Rey *et al.*, Nature 375:291-298, 1995). These include the putative fusion peptide (position 107), the hinge cluster
- 20 (positions 138, 279), the exposed surface of Domain I (positions 176 and 177), and the alpha-helix located in the dimerization Domain II (position 264). Changes at position 107, 138, 176, and 279 were selected early in the passage history, resulting in attenuation of JE SA14-14-2, and remained stable genetic differences from the SA₁₄-14-2 parent (Ni *et al.*, J.
- 25 Gen. Virol. 75:1505-1510, 1994), showing that one or more of these mutations are critical for the attenuation phenotype. The changes at

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positions 177 and 264 occurred during subsequent passage, and appear to be genetically unstable between two SA14-14-2 virus passages in PHK and PDK cells, showing that this mutation is less critical for attenuation.

The nucleotide sequence of the E protein coding region of the RMS
5 was determined to assess potential sequence variability resulting from viral passage. Total RNA was isolated from RMS-infected Vero cells, reversed transcribed, and PCR amplified to obtain sequencing templates. Several primers specific for SA14-14-2 virus were used in individual sequencing reactions and standard protocols for cycle sequencing were
10 performed.

Sequence data revealed two single nucleotide mutations in the RMS E protein, when compared to the published SA14-14-2 JE strain sequence data. The first mutation is silent, and maps to amino acid position 4 (CTT to CTG); the second is at amino acid position 243 (AAA to GAA) and
15 introduces a change from lysine to glutamic acid. Both mutations identified are present in the sequence of the JE wild type strains Nakayama, SA14 (parent of SA14-14-2), and JaOArS982 (Sumiyoshi *et al.*, J. Infect. Dis. 171:1144-1151, 1995); thus, they are unlikely to contribute to virulence phenotype. We conclude that *in vitro* passage in
20 FRhL cells to obtain the RMS did not introduce unwanted mutations in the E protein.

1.7 Structural and Biological Characterization of Chimeric YF/JE Viruses

The genomic structure of chimeric YF/JE viruses recovered from
25 transfection experiments was verified by RT/PCR-based analysis of viral RNA harvested from infected cell monolayers. These experiments were performed to eliminate the possibility that virus stocks were contaminated during transfection procedures. For these experiments, first-pass virus was

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used to initiate a cycle of infection, to eliminate any possible artifacts generated by the presence of residual transfected viral RNA. Total RNA extracts of cells infected with either the YF/JE (prM-E)-SA14-14-2 or YF/JE (prM-E)-Nakayama chimera were subjected to RT/PCR using YF and JE-specific primers that allowed recovery of the entire structural region as two PCR products of approximately 1 kilobase in size. These products were then analyzed by restriction enzyme digestion using the predicted sites within the JE SA14-14-2 and Nakayama sequences that allow differentiation of these viruses. Using this approach, the viral RNA was demonstrated to be chimeric and the recovered viruses were verified to have the appropriate restriction sites. The actual C-prM boundary was then verified to be intact at the sequence level by cycle sequence analysis across the chimeric YF/JE C-prM junction.

The presence of the JE envelope protein in the two chimeras was verified by both immunoprecipitation with JE-specific antisera and by plaque reduction neutralization testing using YF and JE-specific antisera. Immunoprecipitation of ³⁵S-labeled extracts of LLC-MK₂ cells infected with the chimeras using a monoclonal antibody to the JE envelope protein showed that the JE envelope protein could be recovered as a 55 kDa protein, while the same antisera failed to immunoprecipitate a protein from YF-infected cells. Both JE and YF hyperimmune sera demonstrated cross-reactivity for the two envelope proteins, but the size difference between the proteins (YF=53 kDa, unglycosylated; JE=55 kDa, glycosylated) could reproducibly be observed. Use of YF monoclonal antibodies was not satisfactory under the immunoprecipitation conditions, thus, the specificity was dependent on the JE monoclonal antibodies in this analysis.

Plaque reduction neutralization testing (PRNT) was performed on the chimeric viruses and the YF and JE SA14-14-2 viruses using YF and

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JE-specific hyperimmune ascitic fluid (ATCC) and YF-specific purified IgG (monoclonal antibody 2E10). Significant differences in the 50% plaque reduction titer of these antisera were observed for the chimeras when compared to the control viruses in these experiments (Table 3). The YF/JE SA14-14-2 chimeric vaccine candidate, as well as the Nakayama chimera and SA14-14-2 viruses, were neutralized only by JE ascitic fluid, whereas YF 17D was neutralized in a specific fashion by YF ascites and the monoclonal antibody (Table 3). Thus, epitopes required for neutralization are expressed in the infectious chimeric YF/JE viruses, and are specific for the JE virus.

1.8 Growth Properties in Cell Culture

The growth capacity of the chimeras has been examined quantitatively in cell lines of both primate and mosquito origin. Fig. 8 illustrates the cumulative growth curves of the chimeras on LLC-MK₂ cells after low multiplicity infection (0.5 plaque-forming units/cell). In this experiment, YF5.2iv (cloned derivative) and JE SA14-14-2 (uncloned) viruses were used for comparison. Both chimeric viruses reached a maximal virus yield of approximately one log higher than either parental virus. In the case of the YF/JE SA₁₄-14-2 chimera, the peak of virus production occurred 12 hours later than the YF/JE Nakayama chimera (50 hours vs. 38 hours). The YF/JE Nakayama chimera exhibited considerably more cytopathic effects than the YF/JE SA14-14-2 chimera on this cell line.

A similar experiment was carried out in C6/36 cells after low multiplicity infection (0.5 plaque-forming units/cell). Fig. 8 also illustrates the growth kinetics of the viruses in this invertebrate cell line. Similar virus yields were obtained at all points used for virus harvest in

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this experiment, further substantiating the notion that chimeric viruses are not impaired in replication efficiency.

Additional experiments showing the growth properties of RMS are shown in Fig. 9. Briefly, Vero cells were grown in EMEM, 1% L-
5 Glutamine, 1% non-essential amino acid, and 10% FBS buffered with sodium bicarbonate. LLC-MK2 cells were purchased from the ATCC (CLL-7.1, passage 12) and were grown in the same medium as Vero cells. Cells were inoculated with the RMS virus at an MOI of 0.1. Supernatant fluid was sampled at 24 hour intervals for 7 days and frozen at -70°C for
10 subsequent plaque assay. Plaque assays were performed in 6-well plates. The RMS reached more than 8 log₁₀ pfu/ml in 5 days. In LLC-MK2 cells, the RMS grew slower and peaked (6 log₁₀ pfu/ml) at about 6 days.

1.9 *Comparison of Growth Kinetics of the RMS (YF/JE SA14-14-2) with*
15 *YF 17D Vaccine in MRC-5 Cells*

An experiment was performed to assess the ability of the vaccine candidate to propagate in a cell line acceptable for human vaccines. Commercial Yellow Fever 17D vaccine (YF-Vax®) was obtained from
20 Connaught Laboratories, Swiftwater, PA. MRC-5 (diploid human embryonal lung cells) were purchased from ATCC (171-CCL, Batch#: F-14308, passage 18) and grown in EMEM, 2 mM L-Gln, Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 10% FBS.

25 To compare growth kinetics of RMS (sequence appendices 2 and 3; Research Master Seed, YF/JE SA14-14-2; nucleotide sequence of ORF; C: nucleotides 119-421; Pr-M: nucleotides 422-982; E: nucleotides 983-2482; and Non-structural proteins: 2483-10381); (amino acid sequence of ORF; C: amino acids 1-101; Pr-M: amino acids 102-288; E: amino acids 289-

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788; and Non-structural proteins: amino acids 789-3421); (nucleotide sequence of RMS; the coding sequence is from nucleotide 119 to nucleotide 10381)) with YF-Vax®, cells were grown to 90% confluency and infected with RMS or YF-Vax® at an MOI of 0.1 pfu. Since MRC-5
5 cells generally grow slowly, these cells were kept for 10 days post inoculation. Samples were frozen daily for 7-10 days and infectivity determined by plaque assay in Vero cells. YF-Vax® and the YF/JE chimera grew to modest titers in MRC-5 cells (Fig. 10). The peak titer was ~4.7 log₁₀ pfu for YF-Vax® achieved on the second day and was
10 slightly lower, 4.5 log₁₀ pfu, for the RMS after 6 days.

1.10 Growth Curve of YF/JE SA14-14-2 in FRhL cells with and without IFN-inhibitors

Fetal rhesus lung cells were obtained from the ATCC and
15 propagated as described for MRC-5 cells. Growth kinetics of the RMS were determined with and without interferon inhibitors.

Double-stranded RNA appears to be the molecular species most likely to induce interferon (IFN) in many virus infected cells. Induction of interferon apparently plays a significant role in the cellular defense against
20 viral infection. To escape cellular destruction, many viruses have developed strategies to down-regulate induction of interferon-dependent activities. Sindbis virus and vesicular stomatitis virus have been shown to be potent IFN inducers. Using chick embryo cells, mouse L cells, and different viral inducers of IFN, it was shown that 2-aminopurine (2AP)
25 and indomethacin (IM) efficiently and reversibly inhibit IFN action (Sekellick *et al.*, J. IFN Res. 5:651, 1985; Marcus *et al.*, J. Gen. Virol. 69:1637, 1988).

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To test whether inhibition of IFN (if present) in FRhL cells will increase the virus yield, we added 2AP at a concentration of 10 mM or IM at a concentration of 10 mg/ml to the FRhL cells at the time of infection with 0.1 or 0.01 MOI of RMS. Samples were taken daily and frozen for
5 determination of virus infectivity by plaque assay. As shown in Fig. 11A, virus titers peaked on day 4 in the presence or absence of inhibitors. When cells were infected at 0.01 MOI (Fig. 11A), virus titer reached 2.65×10^7 pfu/ml on day 4 in the absence of inhibitors. In cells infected in the presence of IM, virus titer was increased about 2-fold, to 5.95×10^7 pfu/ml
10 on day 4. This increase was more dramatic (4-fold) when 2AP was used (9.7×10^7 pfu/ml). Addition of IM did not increase virus yield when cells were infected at a higher MOI (0.1). A titer of 5.42×10^7 was reached without inhibitor and 3.45×10^7 was achieved in the presence of IM. Addition of 2AP increased virus yields to 1.1×10^8 pfu/ml by day 4 and
15 only 1 log₁₀ pfu was lost in the following 3 days (9.5×10^6 pfu/ml on day 7) (Fig. 11B). We conclude from this experiment that the YF/JE SA14-14-2 vaccine candidate replicates to titers of ~ 7.5 log₁₀/ml in an acceptable cell substrate. The addition of interferon inhibitors can result in a modest increase in yields, but is not a requirement for vaccine production.

20

1.11 Neurovirulence Testing in Normal Adult Mice

The virulence properties of the YF/JE SA14-14-2 chimera was analyzed in young adult mice by intracerebral inoculation. Groups of 10 mice (4 week old male and female ICR mice, 5 each per group) were
25 inoculated with 10,000 plaque-forming units of the YF/JE SA14-14-2 chimera, YF 17D 5.2iv, or the Chinese vaccine strain JE SA14-14-2 and observed daily for 3 weeks. The results of these experiments are illustrated in Fig. 12. Mice receiving the YF5.2iv parent succumbed by

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approximately one week post-inoculation. No mortality or illness was observed among mice receiving either the JE SA14-14-2 parent or the chimera. The inocula used for the experiments were titrated at the time of injection and a subgroup of the surviving mice were tested for the presence
5 of neutralizing antibodies to confirm that infection had taken place. Among those tested, titers against the JE SA14-14-2 virus were similar for animals receiving either this strain or the chimera.

The results of additional experiments investigating the neurovirulence of the YF/JE SA14-14-2 chimera in mice are illustrated in
10 Table 4. In these experiments, all of the mice inoculated with YF5.2iv died within 7-8 days. In contrast, none of the mice inoculated with YF/JE SA14-14-2 died during two weeks of post-inoculation observation.

The results of experiments investigating the neuroinvasiveness and pathogenesis of YF/JE chimeras are illustrated in Table 5. In these
15 experiments, the chimeric viruses were inoculated into 3 week old mice at doses varying between 10,000 and 1 million plaque-forming units via the intraperitoneal route. None of the mice inoculated with YF/JE Nakayama or YF/JE SA14-14-2 died during three weeks of post-inoculation observation, indicating that the virus was incapable of causing illness after
20 peripheral inoculation. Mice inoculated with YF/JE SA14-14-2 developed neutralizing antibodies against JE virus (Fig. 13).

In additional experiments testing the neurovirulence phenotype and immunogenicity of the RMS, 4-week old ICR mice (n=5) were inoculated by the i.c. route with 0.03 ml of graded doses of the RMS or YF-Vax®
25 (Table 6). Control mice received only diluent medium by this route. Mice were observed daily and mortality rates were calculated.

Mice inoculated with YF-Vax® started to die on day 7 (Fig. 14A). The $icLD_{50}$ of unpassaged YF-Vax®, calculated by the method of Reed

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and Muench, was $1.62 \log_{10}$ and the average survival time (AST) at the highest dose ($4.2 \log_{10}$ pfu) was 8.8 days. In contrast, all mice receiving the RMS survived challenge at all doses (Fig. 14B), indicating that the virus is not neurovirulent for mice. None of the mice inoculated with YF-
5 Vax® or the RMS by the peripheral (subcutaneous) route (as shown in Table 6) showed signs of illness or death. Thus, as expected, yellow fever 17D virus was not neuroinvasive.

1.12 Comparison of immunogenicity of YF/JE RMS with YF 17D vaccine

10 The immunogenicity of the of the RMS was compared with that of the YF 17D vaccine in outbred ICR mice. Groups of five 4 week-old mice received graded doses of the vaccines shown in Table 6. Mice were inoculated with 100 μ l of each virus dilution by the s.c. route. For comparison, two groups of mice received two weekly doses of commercial
15 inactivated JE vaccine prepared in mouse brain tissue (JE-Vax®) at 1:30 and 1:300 dilution, representing 10x and 1x the human equivalent dose based on body weight, respectively. Animals were bled 3 and 8 weeks later and neutralizing antibody titers were measured in heat-inactivated sera against homologous viruses by PRNT. End-point titers were the
20 highest dilution of sera which reduced the number of viral plaques by 50% compared to a normal mouse serum control.

The highest N antibody titers were observed 8 weeks after immunization in mice receiving $5 \log_{10}$ pfu of the RMS (Fig. 15 and Table 7). The geometric mean N antibody titer in these mice was 5,614. N
25 antibody responses induced by YF/JE SA14-14-2 vaccine against JE were higher than N antibody responses against YF induced by YF 17D vaccine. Interestingly, the highest concentration of the YF 17D vaccine did not

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induce significant titers of neutralizing antibodies 3 or 8 weeks post immunization, but antibodies were elicited at lower doses.

Very low doses (1.4-2.4 log₁₀ PFU) of YF 17D vaccine elicited an immune response in mice 8 weeks after inoculation (Table 7). This result
5 may indicate delayed replication of the vaccine in mice receiving low virus inocula. In contrast, the YF/JE SA14-14-2 chimeric vaccine in this dose range was not immunogenic. It is likely that the chimeric vaccine is somewhat less infectious for mice than YF 17D. However, when inoculated at an infective dose, the chimera appears to elicits a higher
10 immune response. This may be due to higher replication in, or altered tropism for, host tissues. Animals that received two doses of JE-Vax® did not mount a significant antibody response. Only one animal in the 1:30 dose group developed a neutralizing titer of 1:10 eight weeks after immunization. This might be due to the route (s.c.) and dilution (1:30) of
15 the vaccine.

1.13 Protection of YF/JE SA14-14-2 RMS immunized mice against challenge with virulent JE

The YF/JE SA14-14-2 RMS and other viruses were evaluated for
20 immunogenicity and protection in C57/BL6 mice in collaboration with Dr. Alan Barrett, Department of Pathology, University of Texas Medical Branch, Galveston. Experimental groups are shown in Table 8. Ten-fold dilutions (10²-10⁵) of each virus were inoculated by the s.c. route into groups of 8 mice. Mice were observed for 21 days, at which time
25 surviving animals were bled from the retro-orbital sinus and serum frozen for neutralization tests. The 50% immunizing dose (ID₅₀) for each virus and GMT was determined (see below).

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Surviving mice that received viruses by the s.c. route were challenged on day 28 by i.p. inoculation of 158 LD₅₀ (2,000 PFU) of JE virus (JaOArS982, IC37). Animals were observed for 21 days following challenge. Protection is expressed as the proportion of mice surviving
5 challenge (Table 9).

As expected, YF 17D virus afforded minimal cross-protection against JE challenge. The YF/JE SA14-14-2 RMS chimera was protective at doses $\geq 10^3$ PFU. The 50% protective dose of the chimeric vaccine was 2.32 log₁₀ PFU. Animals that received 3 doses of JE-Vax were solidly
10 protected against challenge. Mice given a single dose of the SA14-14-2 vaccine were poorly protected. Wild-type Nakayama virus was lethal for a proportion of animals, in a dose-dependent fashion; survivors were poorly protected against challenge indicating that the lethal dose was close to the infecting dose for this virus.

15 The YF/JE_{Nakayama} chimeric virus was somewhat more virulent than the Nakayama strain, in that all mice given 10⁵ of the chimera died after inoculation. This is in contrast to earlier studies in outbred mice, in which this virus was not neuroinvasive, confirming the increased susceptibility of C57/BL6 mice to peripheral challenge with JE viruses. Survivors were
20 fully protected against challenge, showing that the infection established by the chimeric virus was more active (immunogenic) than infection by Nakayama virus without the YF replication background. These results show that the combination of viral envelope determinants of a neurovirulent strain (Nakayama) with a replication-efficient virus (YF
25 17D) can enhance virulence of the recombinant, emphasizing the need for genetic stability of the mutations conferring attenuation in the YF/JE_{Nakayama} chimera.

1.14 Serological response

Sera from mice in groups shown in Table 8 were tested 21 days after immunization for neutralizing antibodies. N tests were performed as follows. Six-well plates were seeded with Vero cells at a density of 10^6 cells/well in MEM alpha containing 10% FBS, 1% nonessential amino acids, buffered with sodium bicarbonate. One hundred μ l of each test serum (inactivated at 60°C for 30 minutes) diluted two-fold was mixed with an equal volume of virus containing 200-300 PFU. The virus-serum mixtures were incubated at 4°C overnight and 100 μ l added to each well after removal of growth medium. The plates were overlaid after 1 hour incubation at 37°C with 0.6% agarose containing 3% fetal calf serum, 1% L-glutamine, 1% HEPES, and 1% pen-strep-amphotericin mixed 1:1 with 2x M199. After 4 days of incubation at 37°C, 5% CO₂, a second overlay containing 3% Neutral red was added. After appearance of plaques, the monolayer was fixed with 1% formaldehyde and stained with crystal violet. The plaque reduction titer is determined as the highest dilution of serum inhibiting $\geq$ 50% of plaques compared with the diluent-virus control.

Results are shown in Table 10 and Fig. 16. NT antibody responses in mice immunized with the YF/JE SA14-14-2 chimera showed a dose response and good correlation with protection. At doses of 4-5 logs, the chimeric vaccine elicited higher N antibody responses against JE than either SA14-14-2 virus or wild-type Nakayama virus. Responses were superior to those elicited by YF-Vax® against YF 17D virus. No prozone effect was observed in animals receiving the chimera or infectious-clone derived YF 5.2iv; responses at the highest vaccine dose (5 logs) were higher than at the next lower dose (4 logs). In contrast, mice that received

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SA14-14-2, Nakayama, and YF-Vax® at the highest dose responded less well than animals inoculated with diluted virus.

1.15 Safety and Immunogenicity of ChimeriVax™ in Monkeys

5 The safety of RMS was tested in monkeys, essentially as described in WHO Biological Standards for YF 17D vaccine with minor modifications (see below). Two groups (N=3) of rhesus monkeys were bled and shown to be free from HI antibodies to YF, JE, and SLE. Group 1 received undiluted ChimeriVax™ (Vero-passage 2) by the I.C. route
10 (frontal lobe). Group 2 (N=3) received 0.25 ml of 1:10 diluted commercial YF 17D vaccine (YF-Vax®) by the same route. The virus inocula were frozen, back titrated, and shown to contain 7.0 and 5.0 log₁₀ pfu /0.25 ml of YF/JE SA14-14-2 and YF-Vax®, respectively.

Monkeys were observed daily for clinical signs and scored as in
15 WHO standards. Sera were collected daily for 7 days after inoculations and tested for viremia by plaque assay in Vero cells. Blood collected 2 and 4 weeks post inoculation and tested for NT antibodies to the homologous viruses. None of the monkeys showed sign of illness. Monkeys were euthanized on Day 30, and brains and spinal cords were
20 examined for neuropathology as described in the WHO standards. A sample of the brain and spinal cord from each animal was collected and stored frozen for virus isolation attempts and immunocytochemistry experiments.

As shown in Fig. 17, a low level viremia was detected in all
25 animals in both groups, and lasted for 2-3 days for the RMS and 1-2 days for YF-Vax. All viruses were cleared from the blood by Day 4. According to the WHO standards, monkeys receiving 5,000-50,000 (3.7-

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4.7 log₁₀) pfu should not have viremia greater than 165,000 pfu/ml (approximately 16,500 mLD₅₀). None of the monkeys in the experiments had viremia of more than 15,000 pfu/ml, despite receiving 6 log₁₀ pfu of the RMS.

- 5 Neutralizing antibody titers were measured at 2 and 4 weeks post inoculation (Fig. 18). All monkeys seroconverted and had high titers of neutralizing antibodies against the inoculated viruses. The level of neutralizing antibodies in 2 of 3 monkeys in both groups exceeded a titer of 1:6,400 (the last dilution of sera tested) at 4 weeks post inoculation.
- 10 The geometric mean antibody titers for ChimeriVax were 75 and 3,200 after 2 and 4 weeks respectively and were 66 and 4971 for the YF-Vax® for the same time points (Table 11).

Histopathological examination of coded specimens of brain and spinal cord were performed by an expert neuropathologist (Dr. I.

- 15 Levenbook, previously CBER/FDA), according to the WHO biological standards for yellow fever vaccine. There were no unusual target areas for histopathological lesions in brains of monkeys inoculated with ChimeriVaxTM-JE. Mean lesion scores in discriminator areas were similar in monkeys inoculated with YF-Vax® (0.08) and monkeys inoculated with
- 20 a 100-fold higher dose of ChimeriVaxTM-JE (0.07). Mean lesion scores in discriminator + target areas were higher in monkeys inoculated with YF-Vax® (0.39) than in monkeys inoculated with a 100-fold higher dose of ChimeriVaxTM-JE (0.11). These preliminary results show an acceptable neurovirulence profile and immunogenicity for ChimeriVaxTM-JE vaccine.
- 25 A summary of the histopathology results is provided in Table 22.

1.16 Efficacy of YF/JE Chimera in Protecting Monkeys Against Intracerebral Challenge

The YF/JE chimera were given to adult rhesus monkeys without pre-existing flavivirus immunity by the subcutaneous route. Three
5 monkeys received 4.3 log pfu and three monkeys received 5.3 log pfu of YF/JE SA14-14-2 virus. All 6 monkeys developed very low level (1-2 log/ml) viremias. All animals developed neutralizing antibodies by day 15 (earliest time tested) and titers rose by day 30. Five of six animals survived a very severe intracerebral challenge with a highly virulent JE
10 virus (100,000 mouse LD50 were injected IC 60 days after immunization). None of 4 sham immunized monkeys survived; all died between days 8-10 after challenge. The single death in the immunized group was a pregnant female; pregnancy could have suppressed the cellular immune response to the vaccine. The results show the immunogenicity and protective efficacy
15 of the vaccine, while validating safety with respect to low vaccine viremia. The results of these experiments are illustrated in Tables 12-15.

1.17 Genetic stability of the RMS

The E protein of the attenuated SA14-14-2 virus used to construct
20 the YF/JE chimera differs from its virulent parent (SA14 or Nakayama) at 6 positions; 107, 138, 176, 177, 264, and 279. Because the presence of a single residue controlling virulence would be a disadvantage for any vaccine candidate because of the potential for reversion, studies are being undertaken to determine which residue(s) are responsible for attenuation
25 and in particular whether a single residue is responsible for the difference.

1.18 Position 138 on the E protein

A single mutation of an acidic residue glutamic acid (E) to a basic residue, lysine (K) at position 138 on the E protein of JE virus results in attenuation (Sumiyoshi *et al.*, J. Infect. Dis. 171:1144, 1995). Experiments
5 were carried out to determine whether the amino acid at position 138 of the JE envelope protein (K in the vaccine chimera and E in the virulent Nakayama chimera) is a critical determinant for neurovirulence in mice. Chimeric YF/JE SA14-14-2 (K 138----> E) virus containing the single reversion of

10 K---->E at position 138 was generated from an engineered cDNA template. The presence of the substitution and the integrity of the entire E protein of the resulting virus was verified by RT/PCR sequencing of the recovered virus. A standard fixed-dose neurovirulence test of the virus was conducted in 4-week-old outbred mice by i.c. inoculation with 10^4 pfu
15 of virus. The YF/JE SA14-14-2 and YF/JE Nakayama chimeric viruses were used as controls. The virulence phenotype of YF/JE SA14-14-2 (K-->E) was indistinguishable from that of its attenuated parent YF/JE SA14-14-2 in this assay, with no morbidity or mortality observed in the mice during the observation period (Fig. 19).

20 We conclude that the single mutation at position 138 to the residue found in the JE-Nakayama virus does not exert a dominant effect on the neurovirulence of the YF/JE SA14-14-2 chimera, and that one or more additional mutations are required to establish the virulent phenotype.

25 1.19 Other putative attenuation loci

Additional experiments to address the contributions of the other 6 residues (mentioned above) using the format described here were

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conducted. The mutant viruses constructed by site directed mutagenesis of the YF and JE infectious clones are listed in Table 16. The E proteins of these viruses were sequenced and confirmed to contained the desired mutations. Upon inoculation into weanling mice by the I.C. route it is possible to determine those residues involved in attenuation of the vaccine.

Additional experiments to address the contributions of other residues are underway. The mutant viruses constructed to date by site-directed mutagenesis of the YF and JE infectious clones are listed in Table 16. The methodology is as described above. Results to date confirm that at least two and possibly more than 2 mutations are responsible for the attenuation phenotype of YF/JE SA14-14-2 virus (Table 23).

1.20 Stability of the RMS in Tissue Cultures: Characterization of Genetic Changes, Neurovirulence and Immunogenicity, Serial Passages In Vitro

The RMS was used to inoculate a T75 flask of FRhL2 cells at an m.o.i. of 0.1. Subsequent passages were carried out in T75 flasks and harvested 3 days post-inoculation. At each passage, the culture supernatant was assumed to hold 10^7 pfu/ml and an aliquot corresponding to an moi of approximately 0.1 was added to a fresh flask of cells. The remainder of the culture supernatant was stored at -80°C for later characterization.

1.21 Quasispecies and DNA sequencing

The chimeric JE vaccine is an RNA virus. Selective pressure can cause rapid changes in the nucleic acid sequences of RNA viruses. A mutant virus that invades FRhL cells more rapidly, for example, may gain

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a selective advantage by competing more effectively with the original vaccine virus and take over the culture. Therefore, mutant strains of the vaccine that grow better than the original vaccine may be selected by subculturing *in vitro*. One concern that addressed experimentally is
5 whether such selective pressures might lead to mutant vaccine viruses with increased virulence.

In theory, molecular evolution should occur more rapidly for RNA viruses than DNA viruses because viral RNA polymerases have *higher* error rates than viral DNA polymerases. According to some
10 measurements, RNA virus mutation rates approach one mutation per replication event. This is why an RNA virus can be thought of as a family of very closely related sequences (or "quasispecies"), instead of a single unchanging sequence (a "classical species").

Two different approaches can be taken to determine the sequence of
15 an RNA virus:

1) purify viral genomic RNA from the culture supernatant, reverse-transcribe the RNA into cDNA and sequence this cDNA. This is the approach we have taken. It yields an averaged, or consensus sequence, such that only mutations which represent a large proportion (roughly,
20 >20%) of the viruses in the culture can be detected.

2) Alternatively, cDNA can be cloned and individual clones sequenced. This approach would reveal the quasispecies nature of the vaccine by identifying individual mutations (deviations from the consensus sequence) in some proportion of the clones.

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1.22 *Biological Characterization of Serially Passaged RMS*

As stated above, we demonstrated experimentally that the selective pressures exerted by serial passaging of the RMS does not lead to mutant vaccine viruses with increased virulence. Here, three biological properties of Passages 10 and 18 (P10 and P18) were examined. First, neurovirulence was tested by inoculating mice i.c. with graded doses of P1 as well as P10 and P18. Second, immunogenicity was compared by inoculating mice s.c. with graded doses of the RMS, P10 and P18. Blood was drawn from these mice 30 days post inoculation and serum neutralizing titers were determined and compared. Finally, the growth kinetics of the RMS and of P10 and P18 were compared by inoculating FRhL cells at moi's of 0.1 and 0.01 and collecting samples of culture supernatant daily. The titers in each flask were plotted as a function of time and compared.

15

1.23 *Stability of prM and E genes*

The M and E genes of P10 and P18 were sequenced completely from base 642 to base 2454. Both sequences were identical and carried only one mutation (A-->G) resulting an amino acid substitution from H to R at position 394 on the E protein. This means that selective pressures did not lead to the loss of any of the attenuating mutations of the E gene. Codon H394 (CAC) encodes a Histidine in the RMS but we have found that the second base of this codon is mutated to a G in a significant proportion of the viruses, leading to the expression of Arginine. It is important to emphasize that a mixture of A and G are observed at this position in the sequence data. The ratio of A to G (A/G) was also determined for P1, P4, and P8. Interestingly, the ratio decreases steadily

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from P1 to P10, but at P18 it is back to the value seen at P8. One possible explanation for this observation is that a mutant bearing the H394R mutation gradually became as abundant as the original virus but was then out-competed by a new mutant bearing other mutations not present in the M or E genes and therefore, only detected as a rebound in the A/G ratio. We are reproducing these results by doing a second passaging experiment under identical conditions. It must also be noted that duplicate samples of viral genomic RNA were isolated, reverse-transcribed, amplified, and sequenced in parallel for each passage examined. Reported results were seen in both duplicate samples, arguing against any RT-PCR artifacts obscuring the data.

These observations show that minor genetic changes (one nucleotide substitution in the entire envelope E and M genes) have occurred in the JE sequences of the chimeric vaccine upon passaging, but that selective pressures did not lead to the loss of any of the attenuating mutations of the E gene.

1.24 Neurovirulence phenotype of passages 10 and 18

Groups of five female ICR mice, 3 to 4 weeks-old, received 30 μ l i.c. of undiluted, P1, P10, or P18, as well as 30 μ l of 10-fold dilutions. None of the mice injected with P1, P10, or P18 (doses $\geq 7 \log_{10}$ pfu) showed any sign of illness over a five week period. As determined by back-titration, the doses administered (pfu) were measured as shown in Table 17.

1.25 Immunogenicity of passages 10 and 18

Groups of five female ICR mice were injected subcutaneously (s.c.) with 100 μ l of undiluted virus stock of either the RMS or P10 or P18, as well as with doses of 10^5 and 10^4 pfu (see Table 18, results of back-
5 titration).

1.26 Growth kinetics of passages 10 and 18

Monolayers (90% confluent) of FRhL cells were infected with an moi of 0.1 or 0.01 of RMS, P10, or P18. Time points were then taken
10 daily for seven days and the titer of each time point was determined by plaque assay. Visual observation of cytopathic effects (CPE) on FRhL cells used in this growth curve experiment show that later passages of the RMS have different growth properties than the RMS itself. CPE is clearly greater for P18 and P10 than for the RMS at 4 days postinfection showing
15 that these viruses might replicate much faster than the RMS.

Other observations also show that the growth properties of P10 and P18 differ from those of the RMS. The titers of P1, P10, and P18 are $\sim 2 \times 10^7$, 2×10^8 , and 3×10^8 , respectively. The relative yields of RT-PCR products suggest higher titers of P10 and P18 compared to P1. Although
20 the PCR data are not necessarily quantitative, they are consistent with the observed titers.

These results raise the possibility that we have discovered a variant of the vaccine that is immunogenic, attenuated for mouse neurovirulence, and that grows to titers ten-fold higher than the original vaccine (RMS) in
25 tissue culture. Such a mutant may have value for manufacturing.

Finally, the sequences of the entire genomes of the RMS and p18 were determined and found to be identical, except for the E-H394 mutation (Table 25). There are 6 nucleotide (NT) differences (NT positions are shaded) between the published YF 17D sequences and RMS
5 shown in bold letters. Changes in positions 5461, 5641, 8212, and 8581 are silent and do not result in amino acid substitution, whereas changes in positions 4025 (ns2a) and 7319 (ns4b) result in amino acid substitutions from V to M and from E to K, respectively. Amino acid Methionine (M) at position 4025 is unique for RMS and is not found in any other YF
10 strains, including parent Asibi virus and other yellow fever 17D strains (*e.g.*, 204, 213, and 17DD), whereas Lysine (K) at position 7319 is found in 17D204F, 17D213, and 17DD, but not in 17D204US or Asibi strain. Since the RMS is more attenuated than YF 17D with respect to neurovirulence, and thus has better biological attributes as a human
15 vaccine, it is possible that the amino acid differences at positions 4025 and 7319 in the nonstructural genes of the yellow fever portion of the chimeric virus contribute to attenuation. Other workers have shown that the nonstructural genes of yellow fever virus play an important role in the attenuation of neurovirulence (Monath, "Yellow Fever," *in* Plotkin *et al.*,
20 (Eds.), *Vaccines*, 2nd edition, W.B. Saunders, Philadelphia, 1998).

1.27 Experiment to Identify Possible Interference Between YF 17D and YF/JE SA14-14-2

It is well-established that yellow fever virus encodes antigenic
25 determinants on the NS1 protein that induce non-neutralizing, complement-fixing antibodies. Passive immunization of mice with monoclonal anti-NS1 antibodies confers protection against challenge.

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Active immunization with purified or recombinant NS1 protects mice and monkeys against lethal challenge. The mechanism of protection is presumed to involve antibody-mediated complement-dependent cytotoxicity.

5 In addition to protective determinants on NS1, CTL epitopes on other nonstructural proteins, including NS3, NS2a, and possibly NS5 may be involved in protection. Thus, infection with the YF/JE chimeric virus may stimulate humoral or cellular anti-yellow fever immunity. It is possible, therefore, that use of the chimeric vaccine may interfere with
10 subsequent immunization against YF 17D, or that prior immunization with YF 17D may interfere with seroconversion to YF/JE SA14-14-2. Against this hypothesis is a substantial body of data showing that reimmunization with YF 17D results in a boost in yellow fever N antibodies. Those data show that it should be possible to successfully immunize against JE in an
15 individual with prior YF immunity and vice versa.

To investigate possible interference effects, the experiment shown in Table 19 was initiated. Mice are immunized with one vaccine and subsequently boosted with the heterologous vaccine. Mice are bled every 30 days and sera tested for neutralizing antibodies against heterologous
20 and homologous viruses.

1.28 Seroconversion Rate and Antibody Titers After Primary Immunization

Three groups (n=8) of 3-4 weeks old female outbred ICR mice
25 were immunized with a single dose (5.3 log₁₀ pfu) of ChimeriVaxTMJE (YF/JE SA14-14-2), three groups (n=8) were immunized with two doses of JE-Vax® (0.5 ml of a 1:5 dilution of reconstituted vaccine) and three

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groups (n=8) were immunized with a single dose of YF-Vax® (0.1 ml of a 1:2 dilution of reconstituted vaccine, containing 4.4 log₁₀ pfu, previously determined to induce the highest immune response to YF virus). Six groups (n=4) of mice (similar age, 3-4 weeks old) were kept as controls for
5 booster doses at 3, 6, and 12 months post primary immunization.

All mice were bled 4 and 8 weeks after primary immunization and their neutralizing antibody titers were measured against homologous viruses in a plaque assay. 21/24 (87.5%) of the animals immunized with a single dose of ChimeriVax™-JE developed anti-JE neutralizing antibodies
10 1 month after immunization; at 2 months, 18/24 (75%) were seropositive. Geometric mean increased somewhat between 1 and 2 months post inoculation. In contrast, only 25%-33% of the mice immunized with YF-Vax® seroconverted and antibody responses were low. These results show that YF 17D virus and chimeric viruses derived from YF 17D are
15 restricted in their ability to replicate in the murine host; however, when the envelope of JE virus is incorporated in the chimeric virus, the ability to replicate in and immunize mice is apparently enhanced. Mice receiving two doses of JE-Vax® developed high neutralizing titers against parent Nakayama virus, and titers increased between 1 and 2 months post
20 immunization.

1.29 Secondary Immunization of ChimeriVax™JE and JE-Vax® Immunized Mice With YFVax®

Three months and six months after primary immunization with
25 ChimeriVax-JE, mice were inoculated with YF-Vax® (1:2 dilution of a human dose containing 4.4 log₁₀ pfu). Control mice not previously immunized and of identical age received ChimeriVax™JE only or

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YF-Vax® (Groups 10-13). One month later, mice were tested for presence of YF-specific neutralizing antibodies.

At the 3 month time point, none of the control mice or mice previously immunized with ChimeriVax-JE or JE-Vax seroconverted to YF-Vax®, again confirming the poor immunogenicity of YF-Vax® at the dose used. However, all mice immunized with YF-Vax® 6 months after primary immunization with ChimeriVax-JE and 7/8 mice previously immunized with JE-Vax, seroconverted after immunization with YF-Vax® (Table 24). There was no difference in seroconversion rate or GMT in mice with and without prior immunization with either JE vaccine.

1.30 Secondary Immunization of YF-Vax® Immunized Mice with ChimeriVax™ JE

All mice previously immunized with YF-Vax® and reimmunized with ChimeriVax-JE 3 months later developed neutralizing antibodies to JE (group 7, Table 10). None of the controls seroconverted. Five of 6 mice (83%) previously immunized to YF-Vax® and reimmunized with ChimeriVax-JE 6 months later seroconverted to JE (group 8, Table 10, as did all controls (group 13)), and the GMTs were similar across these groups.

There was no evidence for cross-protection between YF and JE viruses or limitation of antibody response to sequential vaccination with these viruses. Yellow fever 17D vaccine elicits a poor antibody response in the mouse; while this limited interpretation of the data somewhat, it provided a sensitive test of any restriction in replication and immunogenicity of YF 17D virus in mice previously immunized with ChimeriVax-JE. The fact that all mice immunized with ChimeriVax-JE

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responded 6 months later to immunization with YF-Vax® and that the GMT and range of neutralizing antibody titers were similar to controls suggests that the chimeric vaccine imposed no significant barrier to yellow fever immunization.

5

2.0 Construction of cDNA Templates for Generation of Yellow Fever/Dengue (YF/DEN) Chimeric Viruses

Derivation of chimeric Yellow Fever/Dengue (YF/DEN) viruses is described as follows which, in principle, is carried out the same as
10 construction of the YF/JE chimeras described above. Other flavivirus chimeras can be engineered with a similar strategy, using natural or engineered restriction sites and, for example, oligonucleotide primers as shown in Table 20.

15 2.1 Construction of YF/DEN Chimeric Virus

Although several molecular clones for dengue viruses have been developed, problems have commonly been encountered with stability of viral cDNA in plasmid systems, and with the efficiency of replication of the recovered virus. We chose to use a clone of DEN-2 developed by Dr.
20 Peter Wright, Dept. of Microbiology, Monash University, Clayton, Australia, because this system is relatively efficient for regenerating virus and employs a two-plasmid system similar to our own methodology. (See Table 21 for a comparison of the sequences of Dengue-2 and YF/Den-2₂₁₈ viruses; YF/Den-2₂₁₈ contains the nucleotide and amino acid sequences of
25 PUO-218. The NGC and PR-159 strains, which are also listed in Table 21, are other wild strains of dengue that differ from PUO-218 and can be used in the chimeras of the invention.) The complete sequence of this

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DEN-2 clone is available and facilitated the construction of chimeric YF/DEN templates because only a few modifications of the YF clone were required. The relevant steps are outlined as follows.

Similar to the two plasmid system for YF5.2iv and YF/JE viruses,
5 the YF/DEN system uses a unique restriction site within the DEN-2 envelope protein (E) as a breakpoint for propagating the structural region (prM-E) within the two plasmids, hereinafter referred to as YF5'3'IV/DEN (prM-E') and YFM5.2/DEN (E'-E) (see Fig. 20). The two restriction sites for *in vitro* ligation of the chimeric template are *AatII* and *SphI*. The
10 recipient plasmid for the 3' portion of the DEN E protein sequence is YFM5.2(*NarI*[+]*SphI*[-]). This plasmid contains the *NarI* site at the E/NSI junction, which was used for insertion of the carboxyl terminus of the JE E protein. It was further modified by elimination of an extra *SphI* site in the NS5 protein region by silent site-directed mutagenesis. This allowed
15 insertion of DEN-2 sequence from the unique *SphI* site to the *NarI* site by simple directional cloning. The appropriate fragment of DEN-2 cDNA was derived by PCR from the DEN-2 clone MON310 furnished by Dr. Wright. PCR primers included a 5' primer flanking the *SphI* site and a 3' primer homologous to the DEN-2 nucleotides immediately upstream of the
20 signalase site at the E/NSI junction and replacing the signalase site by substitutions that create a novel site, but also introduce a *NarI* site. The resulting 1,170 basepair PCR fragment was then introduced into YFM5.2(*NarI*[+]*SphI*[-]).

The 5' portion of the YF/den-2 clone, including the C/prM junction
25 was engineered by PCR. The C/prM junction was created by incorporating a *TfiI* restriction site at the junction using synthetic oligonucleotides. A 5' PCR fragment encompassing the flanking YF

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sequence 5' untranslated and capsid sequence and a 3' *TfiI* site, together with a 3' PCR fragment beginning with a *TfiI* site at the amino terminus of the dengue-2 prM protein and the flanking dengue-2 prM protein sequence, were ligated into the YF5'3'TV plasmid after intermediate
5 construction in pBluescript. Screening with *TfiI* was used to confirm correct assembly of the chimeric junction in the final plasmid YF5'3'TV/DEN(prM-E).

2.2 Construction of Chimeric YF/DEN Viruses Containing Portions of 10 Two DEN Envelope Proteins

Since neutralization epitopes against DEN viruses are present on all three domains of the E protein, it is possible to construct novel chimeric virus vaccines that include sequences from two or more different DEN serotypes. In this embodiment of the invention, the C/prM junction and
15 gene encoding the carboxyl terminal domain (Domain III) of one DEN serotype (*e.g.*, DEN-2) and the N-terminal sequences encoding Domains I and II of another DEN serotype (*e.g.*, DEN-1) are inserted in the YF 17D cDNA backbone. The junctions at C/prM and E/NS1 proteins are retained, as previously specified, to ensure the infectivity of the
20 double-chimera. The resulting infectious virus progeny contains antigenic regions of two DEN serotypes and elicits neutralizing antibodies against both.

2.3 Transfection and Production of Progeny Virus

25 Plasmid YF5'3'TV/DEN(prME) and YFM5.2/DEN(E'-E) were cut with *SphI* and *AatII* restriction enzymes, appropriate YF and dengue fragments were isolated and ligated *in vitro* using T4 DNA ligase. After

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digestion with XhoI to allow run-off transcription, RNA was transcribed (using 50 ng of purified template) from the SP6 promoter and its integrity was verified by non-denaturing agarose gel electrophoresis. Vero cells were transfected with YF/Den-2 RNA using Lipofectin (Gibco/BRL),
5 virus was recovered from the supernatants, amplified twice in Vero cells, and titrated in a standard plaque assay on Vero cells. The virus titer was 2×10^6 PFU/ml.

2.4 Nucleotide sequencing of YF/Den-2 Chimera

10 Vero cells were infected with YF/DEN-2 (clone 5.75) at an MOI of 0.1. After 96 hours, cells were harvested with Trizol (Life Technologies, Inc.). Total RNA was primed with a YF-5' end NS1 minus oligo, and reverse transcribed with Superscript II RT following a long-RT protocol (Life Technologies, Inc.). Amplification of cDNA was achieved with XL-
15 PCR kit (Perkin Elmer). Several primers specific for dengue type 2 strain PUO-218 were used in individual sequencing reactions and standard protocols for cycle sequencing were performed. Sequence homology comparisons were against the PUO-218 strain prME sequence (GenBank accession number D00345).

20 Sequencing showed that the YF/DEN-2 chimera prME sequence is identical to that of PUO-218 (Gruenberg *et al.*, J. Gen. Virol 69:1391-1398, 1988). In addition, a NarI site was introduced at the 3' end of E, resulting in amino acid change Q494G (this residue is located in the transmembrane domain and not compared in Table 21). In Table 21,
25 amino acid differences in the prME region of YF/Den2 is compared with prototype New Guinea C (NGC) virus and the attenuated dengue-2 vaccine strain PR-159 S1 (Hahn, *et al.*, Virology 162:167-180, 1988).

2.5 Growth Kinetics in Cell Culture

The growth kinetics of the YF/Den-2 chimera were compared in Vero and FeRhL cells (Fig. 16). Cells were grown to confluency in tissue culture flask (T-75). FeRhL cells were grown in MEM containing Earle's salt, L-Glu, non-essential amino acids, 10% FBS and buffered with sodium bicarbonate, and Vero cells were grown in MEM-Alpha, L-Glu, 10% FBS (both media purchased from Gibco/BRL). Cells were inoculated with YF/Den2 at 0.1 MOI. After 1 hour of incubation at 37°C, medium containing 3% FBS was added, and flasks were returned to a CO₂ incubator. Every 24 hours, aliquots of 0.5 ml were removed, FBS was added to a final concentration of 20%, and frozen for determination of titers in a plaque assay. Forty eight hours post infection CPE was observed in FeRhL cells and reached 100% by day 3. In Vero cells, CPE was less dramatic and did not reached 100% by the completion of the experiment (day 5). As shown, the YF/Den2 reached its maximum titer (7.4 log₁₀ pfu/ml) by day 3 and lost about one log (6.4 log₁₀ pfu/ml) upon further incubation at 37°C, apparently due to death of host cells and virus degradation at this temperature. The maximum virus titer in Vero cells was achieved by day 2 (7.2 log₁₀ pfu/ml) and only half log virus (6.8 log₁₀ pfu/ml) was lost on the following 3 days. This higher rate of viable viruses in Vero cells may be explained by incomplete CPE observed in these cells. In sum, the chimera grows well in approved cell substrate for human use.

2.6 Neurovirulence Phenotype in Suckling Mice

Although wild-type unpassaged dengue viruses replicate in brains of suckling mice and hamsters inoculated by the intracerebral route (Brandt *et al.*, J. Virol 6:500-506, 1970), they usually induce subclinical infection and death occur only in rare cases. However, neurovirulence for mice can be achieved by extensive passage in mouse brain. Such neuroadapted viruses can be attenuated for humans. For example, the New Guinea C (NGC), the prototype dengue 2 virus isolated in 1944 and introduced into the Americas in 1981, is not neurovirulent for suckling mice; however after sequential passage in mouse brain it became neurovirulent for mice, but was attenuated for humans (Sabin, Am. J. Trop. Med. Hyg., 1:30-50, 1952; Sabin *et al.*, Science 101:640-642, 1945; Wisseman *et al.*, Am. J. Trop. Med. Hyg. 12:620-623, 1963). The PUO-218 strain is a wild type dengue 2 virus isolated in 1980 epidemic in Bangkok. It is closely related to the NGC strain by nucleotide sequencing (Gruenberg *et al.*, J. Gen. Virol 69:1391-1398, 1988). When the prME genes of the PUO-218 strain were inserted into the neuroadapted NGC backbone, the chimeric virus was attenuated for 3-days old mice inoculated by the I.C. route (Peter Wright, Xth International Congress of Virology, Jerusalem, Israel, 1996). The PUO218 virus differs from NGC in one amino acid in prM (residue 55 is F in NGC and is L in PUO218) and 6 amino acids in the E protein (71 D->E, 126K->E, 141I->V, 164 I->V, 402I->F, and 484 V->I) (see Table 21). All amino acid differences (except residue E-126) are also present in PR S1 strain (attenuated vaccine strain), indicating that they may not be involved in attenuation. Only residue 126 on the E protein is different between these viruses. This residue was shown to be responsible for the neurovirulent phenotype of the mouse adapted NGC (Bray *et al.*, J. Virology 72:1647-1651, 1998).

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Although mouse neurovirulence does not predict virulence/attenuation of dengue viruses for humans, it is important to determine the neurovirulence of a YF/Den-2 chimeric virus. YF 17D retains a degree of neurotropism for mice, and causes (generally subclinical) encephalitis in monkeys after

5 IC inoculation. For vaccine development of a den/YF chimera it will be necessary to show that the construct does not exceed YF 17D in neuroinvasiveness and neurovirulence. Ultimately safety studies in monkeys will be required. In initial studies, we determined if insertion of the prME of the PUO218 into YF 17D vaccine strain will affect its

10 neurovirulence for suckling mice (Table 24). Groups of 3, 5, 7, and 9 days old suckling mice were inoculated by the I.C. route with 10,000 pfu of YF/Den-2 or YF/JE SA14-14-2 chimera and observed for paralysis or death for 21 days. For controls similar age groups were inoculated either sham with medium (I.C. or I.P.) or with 1,000 pfu of unpassaged

15 commercial YF vaccine (YF-Vax) by the I.P. route (it is not necessary to inoculate suckling mice with YF-Vax by the I.C. route because we have previously shown that this vaccine is virulent for 4-weeks old mice by this route).

As shown in Fig. 22, all suckling mice (3 to 7 days old) inoculated

20 by the I.C. route with the YF/Den2 chimera died between 11 and 14 days post inoculation, whereas 8 out of 10 suckling mice (9 days old) survived. Similarly, all suckling mice (3-5 days old) inoculated with YF-Vax by the I.P. route, with a dose which was 10-fold lower than the YF/Den2 chimera, died between 11 to 13 days post inoculation (Fig. 23). All nine

25 day old, as well as 8 out of 9 seven day old, mice inoculated with the YF-Vax survived. Similar results to the YF/Den2 chimera obtained with suckling mice inoculated with the YF/JE SA14-14-2 chimera.

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As is mentioned above, when prME genes of the PUO218 strain were inserted into the NGC backbone the chimeric virus was not neurovirulent for 3 days old suckling mice inoculated by the I.C. route. In contrast, when these genes were inserted into the 17D backbone, the
5 resulting YF/Den2 chimera demonstrated a neurovirulence phenotype (for suckling mice) similar to the YF/ JE SA14-14-2. This experiment also demonstrated that the replacement of the prME genes of the YF 17 D with prME genes of the Dengue 2 PUO218 resulted in a chimeric virus which was less neurovirulent than the 17D parent strain.

10 Unlike most flaviviruses, there is no correlation between neurovirulence of dengue viruses in mice and humans. Currently the most suitable animal models for dengue infection are Old World monkeys, New World monkeys, and apes that develop subclinical infection and viremia. There is, however, no animal model for the most severe illness (DHF) in
15 humans, which occurs when individuals become infected with a heterologous serotype due to antibody dependent enhancement of infection. Today it is generally accepted that a tetravalent vaccine is required to induce protective immunity in human beings against all four serotypes to avoid sensitizing vaccinee to more severe illness DHF. For
20 the last fifty years, many approaches have been undertaken to produce effective dengue vaccines and although dengue viruses have been satisfactory attenuated (*e.g.*, PR-159/S-1 for Dengue 2) in many cases *in vitro* or *in vivo* correlation of attenuation were not reproducible in humans. A current strategy is to test selected live virus vaccine candidates stepwise
25 in small numbers of human volunteers. Many laboratories around the world are exploring various strategies to produce suitable vaccine candidates. These range from subunit vaccines including prME (protein vaccine or DNA vaccine) of dengue viruses to live attenuated whole

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viruses (produced by tissue culture passage or recombinant DNA technology). Although some of these candidates have shown promise in preclinical and human volunteers, development of a successful dengue vaccine remained to be implemented.

- 5 Evaluating the immunogenicity and protective efficacy of the YF/Den2 chimera in monkeys should shed light on selection of appropriate prME genes (from wild type or attenuated strain) for construction of all 4 serotypes of chimeric dengue viruses.

10 2.7 *Stability of prME genes of ChimeriVax™-D2 virus in vitro*

- The ChimeriVax™-D2 virus at passage 2 post transfection was used to inoculate a 25 cm² flask of Vero cells. Total RNA was isolated and the complete nucleotide sequence of the ChimeriVax™-D2 was determined (P3) and compared to the published sequence of the YF 17D virus (Rice *et al.*, Science 229:726-733, 1985). There was one nucleotide difference: at position 6898 there was an A in the chimera (P3), which was a C in the 17D nucleotide sequence. No difference in the prME region was found when the sequence of ChimeriVax™-D2 was compared to its parent dengue 2 virus (PUO218 strain). Also, no mutations were found in
- 15 virus (Rice *et al.*, Science 229:726-733, 1985). There was one nucleotide difference: at position 6898 there was an A in the chimera (P3), which was a C in the 17D nucleotide sequence. No difference in the prME region was found when the sequence of ChimeriVax™-D2 was compared to its parent dengue 2 virus (PUO218 strain). Also, no mutations were found in
- 20 the prME genes of the chimera upon 18 passages in VeroPM cells. Within the YF genes, however, there was one silent mutation in position 6910 (C to A), and at position 3524 the P18 virus appeared to be heterozygous (both parent nucleotides, G and mutant A, were present). This would translate into a mixture of E and K amino acids at position 354 of the NS1
- 25 protein.

 Similar to the passage 3 virus, the passage 18 virus was not neurovirulent for 4 week old outbred mice inoculated by the IC route (5

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log₁₀ pfu was the highest dose tested). Passage 3, passage 5, passage 10, and passage 18 of ChimeriVax™-D2 were inoculated into mice by SC and IC routes, and antibody responses were compared. There were no significant differences in production of anti-dengue 2 neutralizing antibodies across 18 passages (Table 26).

2.8 Viremia, immunogenicity, and protective efficacy of ChimeriVax™-D2 in YF immune monkeys

Because ChimeriVax™-viruses contain core and NS genes of the YF 17D virus, it is important to determine if preimmunity to the 17D vaccine interferes with vaccination with ChimeriVax™-D2 virus. As is discussed above, in the case of ChimeriVax™-JE, there was no significant interference between YF17D immunity and ChimeriVax™-JE virus, measured by production of neutralizing antibodies in mice.

Since YF17D vaccine and both ChimeriVax™-JE and ChimeriVax™-D2 were not highly immunogenic in mice inoculated by the SC route (see also table 26), non-human primates were used, which are more susceptible/relevant for evaluation of flavivirus vaccines for humans. Sixteen rhesus monkeys (some of which were previously immunized with YF 17D vaccine) received ChimeriVax™-D2, YF 17D vaccine, or a wild type dengue 2 virus (strain S16803). As is shown in Table 27, all YF immune monkeys seroconverted to the YF/dengue 2 or wild type dengue 2 virus, demonstrating a lack of vector immunity. These monkeys were also protected from viremia after challenge with wild type dengue 2 virus. In contrast, YF 17D immunized monkeys, as well as non-immunized animals, became viremic after challenge with wild type dengue 2 virus. Wild type dengue 2 viruses produce a high level of viremia (3-5 logs) in

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rhesus monkeys, which lasts between 3-6 days. Attenuation of dengue 2 viruses can therefore be estimated by comparing the level and duration of viremia with reference wild-type strains. These experiments clearly showed that core and non-structural proteins of YF 17D virus present in
5 ChimeriVaxTM-D2 do not interfere with ChimeriVaxTM-D2 immunization.

2.9 Dose response effectiveness of ChimeriVaxTM-D2 in monkeys.

The goals of this experiment were to (i) determine the viremia profile of the vaccine candidate, using YF 17D and wild type dengue 2
10 virus controls, (ii) compare neutralizing antibody responses to the vaccine candidate and wildtype virus, and (iii) determine minimum dose required for protection against challenge with wild type dengue-2 virus. It was anticipated that these experiments would define the viremia profile of the ChimeriVaxTM-D2 virus in non-YF immune monkeys, and would
15 determine whether immunization with a single dose results in protection of animals against challenge with a wild type dengue 2 virus. Protection in these experiments is defined as reduction of viremia in test monkeys compared to control viruses.

As is shown in table 28, all monkeys became viremic, and the
20 duration of viremia was dose-dependent. The peak level of viremia for ChimeriVaxTM-D2 was between 1.3 to 1.6 log₁₀ pfu, which was significantly lower than that of the wild type dengue virus (3.6 log₁₀ pfu).

All monkeys developed anti-dengue 2 neutralizing antibodies by day 15. Lower dose of the vaccine resulted in lower GMTs, however, by
25 day 30 post-immunization, all monkeys developed high titers of neutralizing antibodies, independent of the dose they received. Upon challenge, no viremia was detected in any immunized monkeys,

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demonstrating that even at its lowest dose ($2 \log_{10}$ pfu), ChimeriVaxTM-D2 had protected these animals from dengue infection (Table 29).

2.10 Growth characteristics of ChimeriVaxTM-JE virus in *Culex*

5 *tritaeniorhynchus*, *Aedes albopictus*, and *Aedes aegypti* mosquitoes

As is described above, ChimeriVaxTM-JE, which consists of a YF 17D virus backbone containing the prM and E genes from the JE vaccine strain SA14-14-2, exhibited restricted replication in non-human primates, producing only a low level viremia following peripheral inoculation.

- 10 Although this reduces the likelihood that hematophagous insects could become infected by feeding on a vaccinated host, it is prudent to investigate the replication kinetics of the vaccine virus in mosquito species that are known to vector the viruses from which the chimera is derived. In this study, ChimeriVaxTM-JE virus was compared to its parent viruses
- 15 (YF17D and JE SA14-14-2), as well as to wild type JE SA14 virus, for its ability to replicate in *Culex tritaeniorhynchus*, *Aedes albopictus*, and *Aedes aegypti* mosquitoes. Individual mosquitoes were exposed to the viruses by intrathoracic (IT) virus inoculation or by oral ingestion of a virus-laden blood meal.

20

2.11 Intrathoracic inoculation

- Mosquitoes were inoculated 7-10 days post-emergence with 0.34 ml of approximately $6.0 \log_{10}$ pfu/ml virus suspension ($\sim 5.5 \log_{10}$ pfu/mosquito). This route of inoculation was chosen to avoid variables,
- 25 such as threshold titer, that might limit midgut infection and subsequent dissemination of the viruses. Three mosquitoes per virus were collected either at 24 hour intervals for 5-10 days or at 72 hour intervals for 18 days.

Individual mosquitoes were triturated in 1 ml of M199 media (Gibco BRL, Grand Island, New York) supplemented with 5% fetal calf serum, clarified by brief centrifugation, and then titrated in Vero cells to monitor virus replication.

- 5 Both JE SA14 and JE SA14-14-2 viruses replicated in *Cx. tritaeniorhynchus* following IT inoculation, reaching titers at day 14 of 6.7 and 6.0 log₁₀ pfu/mosquito, respectively (Figure 24A). Additionally, IFA conducted on head squashes from JE SA14 and JE SA14-14-2-inoculated *Cx. tritaeniorhynchus* mosquitoes was positive for detection of JE virus
- 10 antigen. In contrast, YF 17D and ChimeriVax™-JE did not replicate in *Cx. tritaeniorhynchus* mosquitoes. Virus titers declined rapidly following inoculation, and no virus was detectable by plaque titration assay in YF 17D or ChimeriVax™-JE-inoculated mosquitoes by days 1 and 2, respectively (Figure 24A). IFA analysis of head squashes from *Cx. tritaeniorhynchus* mosquitoes inoculated with ChimeriVax™-JE or YF
- 15 17D was negative for JE or YF virus antigens, supporting our observation that neither the chimera nor YF 17D replicate in this mosquito species.

- ChimeriVax™-JE did replicate in IT-inoculated *Ae. albopictus* mosquitoes, reaching a titer of 5.2 log₁₀ pfu/mosquito at day 18 (Figure
- 20 24B) and IFA results were weakly positive for both JE virus and YF virus antigens. The JE SA14 and JE SA14-14-2 viruses also replicated in *Ae. albopictus* mosquitoes, reaching maximum titers of 6.3 and 6.0 log₁₀ pfu/mosquito, respectively. YF 17D virus did not replicate to high titers in *Ae. albopictus* mosquitoes, however, a low level of detectable virus was
- 25 maintained (3.8 log₁₀ pfu/mosquito at day 18) (Figure 24B) and IFA-stained head squashes were weakly positive for YF virus antigen. ChimeriVax™-JE and YF 17D inoculated IT into *Ae. aegypti* mosquitoes

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replicated at low levels over the 18 day incubation period (Figure 24C). Peak titers of 3.6 and 4.4 log₁₀ pfu/mosquito, respectively, were reached on day 15. IFA staining of head tissues from ChimeriVax™-JE and YF 17D IT-inoculated mosquitoes was weakly positive. Both JE SA14 and JE
5 SA14-14-2 viruses proliferated in *Ae. aegypti* mosquitoes, reaching peak titers of 6.3 and 6.1 log₁₀ pfu/mosquito on days 9 and 18, respectively.

2.12 Oral infection

Seven to ten day old *Ae. albopictus* and *Ae. aegypti* mosquitoes
10 were orally exposed to an artificial virus-containing blood meal that was prepared from equal parts of washed calf red blood cells (Colorado Serum Company, Denver, Colorado) and freshly harvested virus. The blood/virus mixture was heated to 37°C immediately prior to feeding. Mosquitoes were starved for 48-72 hours prior to feeding on virus/blood soaked cotton
15 pledgets. *Cx. tritaeniorhynchus* mosquitoes are reluctant to feed from blood-soaked pledgets, and were therefore fed using a membrane feeder. Mosquitoes were allowed to feed for 15-30 minutes, after which fully engorged mosquitoes were collected. Three mosquitoes per virus were harvested at 48-72 hour intervals over a 15-18 day period, or, in a second
20 experiment, all mosquitoes were harvested at 22 days after feeding.

Figure 25A illustrates growth of the viruses in orally exposed *Cx. tritaeniorhynchus* mosquitoes. Individuals that were fed a blood meal containing 6.9 log₁₀ pfu/ml ChimeriVax™-JE virus did not become infected. Similar results were observed in mosquitoes that had ingested a
25 blood meal containing YF 17D virus. In contrast, high virus titers were detected in *Cx. tritaeniorhynchus* mosquitoes that had ingested JE SA14 or JE SA14-14-2 viruses.

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Figures 25B and 25C illustrate growth of the viruses in orally exposed *Ae. albopictus* and *Ae. aegypti* mosquitoes, respectively. Only JE SA14 and JE SA14-14-2 viruses successfully infected and replicated in these species. For example, in *Ae. aegypti* mosquitoes on day 15, the titers of JE SA14 and JE SA14-14-2 viruses were 5.4 and 5.5 log₁₀ pfu, respectively. In contrast, mosquitoes that had ingested 4.7 log₁₀ pfu/mosquito of YF17D virus or 4.5 log₁₀ pfu/mosquito of ChimeriVaxTM-JE virus failed to become infected.

In a separate experiment, *Ae. aegypti* and *Ae. albopictus* mosquitoes were orally exposed to JE SA14-14-2, YF 17D, and ChimeriVaxTM-JE viruses and processed after 22 days extrinsic incubation to permit growth to maximum virus titers. The results of this experiment are summarized in Table 30. Only JE SA14-14-2 virus was detectable in mosquitoes. Because ChimeriVaxTM-JE did not grow in any of the mosquito species tested, transmission studies were not performed.

Viruses recovered from *Ae. Albopictus* after IT or oral inoculation, or from *Ae. Aegypti* after IT inoculation, were identical to their parent ChimeriVaxTM-JE virus (Vero2FrhL1) in the prME region.

2.13 Amplification and sequencing of the "late replicating" ChimeriVaxTM-JE viruses isolated from mosquitoes

Ae. albopictus mosquitoes inoculated with ChimeriVaxTM-JE by IT or oral routes and *Ae. aegypti* inoculated with ChimeriVaxTM-JE by IT route, were harvested on day 15 post-inoculation. After triturating in 1 ml of M199 (supplemented with 5% fetal calf serum), samples were clarified by centrifugation, filtered through a 0.2 micron filter, and used to inoculate a T-25 cm² flask of VeroPM cells, passage 144 (0.5 ml/flask). After 1

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hour, virus adsorption at 37°C, 5 ml MEM-containing 5% FBS was added and flasks returned to the 37°C CO₂ incubator. Viruses were harvested from supernatants 4 days later (at 2+ CPE) and kept frozen at -70°C.

Total RNA was extracted from infected monolayers by the use of the

- 5 Trizol® reagent (Gibco/BRL), reverse-transcribed, amplified by XL PCR (Perkin-Elmer), and the prME region was sequenced. Viruses recovered from *Ae. Albopictus* after IT or oral inoculation, or from *Ae. Aegypti* after IT inoculation, were identical to their parent ChimeriVax™-JE virus (Vero2FrhL1) in the prME region.

10

2.14 Growth characteristics of ChimeriVax™-D2 virus in Aedes albopictus and Aedes aegypti mosquitoes

Similar experiments were carried out in *Ae. albopictus* and *Ae. aegypti* mosquitoes with ChimeriVax™-D2 virus. For controls, the

- 15 YF17D vaccine and a dengue 2 wild type virus were used. Dengue 2 wild type virus grew to more than 5 log₁₀ pfu/ml in both mosquito species inoculated by IT or oral routes. The growth of YF17D vaccine was lower than the wild type dengue 2 virus, and did not exceed 4 log₁₀ pfu/ml. Interestingly, the ChimeriVax™-dengue 2 virus revealed the most
20 restricted growth in both mosquito species inoculated by either route (its titer did not exceed 3 log₁₀ pfu/ml) (Figure 26).

2.15 Summary of JE and Den2 Experiments in Mosquitos

- In summary, ChimeriVax™-JE virus did not replicate following
25 ingestion by any of the three mosquito species. Additionally, replication was not detected after IT inoculation of ChimeriVax™-JE in the primary JE virus vector, *Cx. tritaeniorhynchus*. ChimeriVax™-JE exhibited

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moderate growth following IT inoculation into *Ae. aegypti* and *Ae. albopictus* mosquitoes, reaching titers of 3.6-5.0 log₁₀ pfu/mosquito. There was no change in the virus genotype associated with replication in mosquitoes. Similar results were observed in mosquitoes of all three
5 species that were IT inoculated or had orally ingested the YF 17D vaccine virus. In contrast, all mosquitoes either IT inoculated with, or orally fed, wild type and vaccine JE viruses became infected, reaching maximum titers of 5.4-7.3 log₁₀ pfu/mosquito. The growth of ChimeriVaxTM-D2 in both *Ae. albopictus* and *Ae. aegypti* mosquitoes inoculated by IT or oral
10 routes was also significantly lower than its parent wild type dengue 2 and YF17D vaccine viruses.

These results showed that ChimeriVaxTM-JE and ChimeriVaxTM-D2 viruses are restricted in their abilities to infect and replicate in these mosquito vectors. The low viremia caused by the viruses in primates and
15 poor infectivity for mosquitoes are safeguards against secondary spread of the vaccine virus.

3.0 Construction of ChimeriVaxTM YF/DEN-1

A yellow fever/dengue 1 (YF/DEN-1) chimeric virus was
20 constructed using a novel technology, which differs from the approaches used to construct Yellow fever/Japanese encephalitis (YF/JE) chimeric viruses as described by Chambers *et al.* (J. Virol. 73:3095-4101, 1999; see above), and the construction of YF/DEN-4 chimera (see below). We used the same two plasmid system used to create YF/DEN-4. These plasmids
25 first encoded the yellow fever (YF) genome as created by Rice *et al.* (New Biol. 1:285-296, 1989). Later, the structural membrane precursor and envelope protein genes, *i.e.*, the prME region, of the YF genome plasmids

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was replaced with those of the JE SA14-14-2 sequence, and the resulting plasmids were used to produce RNA *in vitro*, which was then transfected into cells to produce live YF/JE chimeric virus. Although the two-plasmid system was suitable for the construction of JE, DEN-2, and DEN-4
5 chimeras in a YF backbone, the marked instability of one of the plasmids created with DEN-1 sequences was such that we opted for a PCR alternative to replace it.

Here we describe in detail the procedures for construction of the YF/DEN-1 chimera (Figs. 27 and 28). Dengue 1 (strain PUO359 isolated
10 in 1980 in Thailand) was passed once in C6/36 and total RNA was isolated. The dengue 1 prME region was first amplified and sequenced using primers derived from consensus sequences (Genbank). The sequence data created was applied to primer design and was used, with the cDNA produced earlier, as a PCR starting point for assembly of chimeric
15 YF/DEN-1 virus. A dengue 1 PCR product encoding prM and the 5' end of E was then used as a template, along with template encoding the capsid (C) of yellow fever derived from plasmid pYF5'3'IV/JE SA 14-14-2, in an overlap extension PCR to result in a single fusion product, which was then cloned into a vector fragment of pYFM5'3'IV in which JE sequences were
20 deleted. In contrast to the construction of the YF/DEN-4 (see below) and YF/JE SA 14-14-2 (see above) plasmid systems, the 3' end of the DEN-1 envelope was fused to the YF non-structural genes normally present in the pYFM5.2/JE SA 14-14-2 plasmid using an overlap extension PCR similar to that used to construct the fusion of YF capsid to the DEN-1 prM and
25 envelope gene 5' end. Next, the pYD1-5'3' plasmid was transformed into *E. coli* strain MC1061 (RecA-) for amplification, followed by midi-scale plasmid purification, while the DEN-1Env/YF5.2 (Fragment H) was gel purified. *In vitro* ligation of the plasmid to the PCR product resulted in

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full-length virus cDNA template for RNA transcription. All steps involving cDNA fragments, plasmids, and PCR products were carried out in a BL-2 lab designated for recombinant DNA work. Steps involving manipulations of infectious RNA and virus were carried out in a limited
5 access BL-2+ virus lab.

3.1 Amplification of Dengue 1 sequence

Dengue 1 cDNA was synthesized from RNA using the Superscript II™ method. All primers for this experiment were synthesized by Life
10 Technologies and are listed in Table 31. Upon arrival as lyophilized material, they were dissolved to 250 µM stock solutions using RODI-water. From this, 25 µM working solutions were made. The fragment encoding the SP6 promoter and the yellow fever capsid (Fragment A) was amplified using XL-PCR Reaction Kit™
15 (Perkin-Elmer Part#N808-0192), with 0.5 µl (250 ng) of pYF5'3'TV plus 3.5 µl RODI-water as template and primers 1 and 2 (see Table 31). The fragment encoding dengue 1 prM and 5' end of E (Fragment B) was amplified using the XL-PCR Reaction Kit™ (Perkin-Elmer Part#N808-0192) and primers 3 and 4. The fragment encoding the 3' end
20 of the Dengue 1 envelope gene (Fragment F) was amplified using the same protocol, but with primers 5 and 7. The fragment encompassing the YF portion of pYFM5.2 (Fragment G) was amplified using the same protocol, but with primers 8 and 9 and 1 µl of pYFM5.2/2 with 39 µl water. The PCR for fragments F and G required an annealing temperature of 50°C
25 and an extension time of 6.5 minutes. The PCR reaction was performed using the following master mixes for each reaction.

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Upper Mix (UM)

3.3 x buffer	18 μ l
RTth	1.25 μ l
H ₂ O	1 μ l
Volume	20.25 μ l

Lower Mix (LM)

3.3 x buffer	12 μ l
10 mM dNTPs	8 μ l
5' primer	1 μ l
3' primer	1 μ l
Mg(OAc) ₂	4 μ l
H ₂ O	14 μ l
Volume	40 μ l

cDNA Mix

H ₂ O	36 μ l
Dengue 1 cDNA	4 μ l
Volume	40 μ l

The LM was added to a Perkin-Elmer thin-walled 0.2 ml tube. Next, Ampliwax 100 (Perkin-Elmer) was added to the tube, which was then placed in a Perkin-Elmer 2400 Thermal Cycler and heated to 80°C for 5 minutes, and then cooled to 4°C. The cDNA and UM were then added to the top of the wax layer. The tube was then cycled in a Perkin-Elmer 2400 as follows: 94°C, 1 minute; repeat 30x (94°C, 15 seconds; 53°C, 15 seconds; 68°C, 3 minutes), 72°C, 4 minutes; 4°C, hold. The expected sizes for the fragments are as follows.

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Fragment	Approximate Size (kb)
A	0.94
B	0.65
F	1.3
G	6.0

Forty μ l of each fragment was then separated on a 1% Agarose/TAE gel and purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704). Next, the concentrations of the purified fragments were determined by UV absorption using 1:40 dilutions in RODI-water.

Sample	A280	A260	280/260	260/280	Concentration
Fragment A	0.0116	0.0260	0.4453	2.2457	52 ng/ μ l
Fragment B	0.0076	0.0202	0.3782	2.6440	40.4 ng/ μ l
Fragment F	0.0160	0.0335	0.4785	2.0898	67 ng/ μ l
Fragment G	0.0199	0.0380	0.5242	1.9076	76 ng/ μ l

3.2 Recombinant PCR

To create a fusion between the yellow fever capsid and DEN-1 prM, a recombinant PCR technique known as overlap-extension PCR was used to create Fragment E. The same basic UM and LM were used, and primers 1 and 4 replaced earlier primers. The same approach was used to create a fusion between fragment F and G, resulting in fragment H. For this, primers 5 and 9 were used. The cDNA mixes were as follows:

	Fragment E	Fragment B control	Fragment A control
H ₂ O	37.82 μ l	38.97 μ l	38.85 μ l
Fragment A	1.15 μ l	0 μ l	1.15 μ l
Fragment B	1.03 μ l	1.03 μ l	0 μ l
Volume	40 μ l	40 μ l	40 μ l

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	Fragment H	Fragment F control	Fragment G control
5 H ₂ O	38.9 µl	39.7 µl	39.2 µl
Fragment F	0.3 µl	0.3 µl	0 µl
Fragment G	0.8 µl	0 µl	0.8 µl
Volume	40 µl	40 µl	40 µl

The same protocol that was used for creation of fragments A, B, F,
 10 and G was used, except that only ½ the cDNA, UM, and LM were used for
 the control reactions. The tubes were cycled in a Perkin-Elmer 2400 as
 follows: 94°C, 1 minute; repeat 30x (94°C, 15 seconds; 55°C, 15 seconds;
 68°C, 2 minutes; 72°C, 7 minutes; 4°C, hold, for Fragment C and its
 controls. For Fragment H and its controls, the annealing temperature was
 15 50°C and the extension time was 6.5 minutes. The expected sizes were as
 follows:

Fragment	Approximate Size (kb)
E	1.59
H	7.3

20

Forty µl of Fragment E and 50 µl of Fragment H was then separated
 on a 1% Agarose/TAE gel and purified using the QIAquick Gel Extraction
 Kit (Qiagen cat#28704). Next, the concentration of the purified fragment
 was determined by UV absorption using 1:40 dilutions in RODI-water.

25	Sample	A280	A260	280/260	260/280	Concentration
	Fragment E	0.0334	0.0516	0.6473	1.5449	103.2 ng/µl
	Fragment H	0.0035	0.0068	0.5088	1.9655	13.6 ng/µl

3.3 Cloning of Fragment E into the Yellow Fever Vector

The capsid-prME fusion was cloned into the yellow fever plasmid needed, and after digestion of the purified Fragment E, as well as pYF5'3'TV, with the appropriate enzymes. The digested plasmid resulted in two bands. Lower bands seen contain a fragment of Japanese

- 5 encephalitis virus equivalent to Fragment E. All restriction enzymes, buffers, and 100x BSA were from New England Biolabs. All the digestions were incubated in a Perkin-Elmer 480 cyclor set to hold at 37°C overnight.

Fragment E Digest

10	Fragment E (600 ng)	5.8 µl
	NEB Buffer 4	4 µl
	10x BSA	4 µl
	H ₂ O	24.2 µl
	Not I	1 µl
15	Nhe I	1 µl
	Volume	40 µl

pYFMIV5'3' Digest

20	pYFMIV5'3' (1.02 µg)	2 µl
	NEB Buffer 4	2 µl
	10x BSA	2 µl
	H ₂ O	12 µl
	Not I	1 µl
	Nhe I	1 µl
25	Volume	20 µl

3.4 Vector Dephosphorylation

Calf Intestinal Phosphatase (CIP) from New England Biolabs (cat#290S) was diluted 1:10 in 1x CIP Buffer. One µl of this dilution was

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then added to the pYFMIV5'3' digest, and incubated for 1 hour at 37°C. Then, 0.8 µl 125 mM EDTA was added to the tube, which was placed at 75°C for 10 minutes to deactivate CIP.

5 3.5 Gel Excision

The digested fragment E and the digested plasmid were separated on a 1.0% Agarose/TAE gel, and were purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704).

10 3.6 Ligations

The digested Fragment E and pYF5'3'IV were ligated using T4 DNA Ligase (New England Biolabs cat#202S) to create pYD1-5'3'. All ligation reactions were incubated in a Perkin-Elmer 480 cyclor set to hold at 16°C overnight.

15 PYD1-5'3' Ligation

Fragment E (97.5 ng)	12.8 µl
pYFM5'3' (50 ng)	3.0 µl
H ₂ O	1.2 µl
10x T4 ligase buffer	2 µl
20 T4 DNA ligase	1 µl
Volume	20 µl

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pYFM-5'3' Control Ligation

Fragment E (97.5 ng)	0 µl
pYFM5'3' (50 ng)	3.0 µl
H ₂ O	14 µl
10x T4 ligase buffer	2 µl
T4 DNA ligase	1 µl
Volume	20 µl

3.7 Transformations

- 10 Ligation reactions were individually transformed into *E. coli* strain MC1061 (recA-). Briefly, an aliquot of MC1061 was removed from storage at -80°C and allowed to thaw on ice for one to two minutes. 0.9 ml of cold 0.1 M CaCl₂ was added to the cells. One hundred µl of cells was aliquoted into three 12 ml culture tubes on ice. Ten µl of each
- 15 ligation reaction was added to each culture tube, leaving the third tube as a no DNA control. Culture tubes were left on ice for 30 minutes. The tubes were heat shocked in a water bath at 42°C for 45 seconds, and then were put back on ice for 2 minutes. 0.9 ml SOC medium was added to each culture tube and incubated at 225 pm in a shaking incubator at 37°C for 1
- 20 hour. Each transformation mix was aliquoted into 1.5 ml microcentrifuge tubes. One hundred µl of each mix was spread onto LB/Agar-Amp (100 µg/ml) plates and labeled as "neat." Each tube was spun at 14,000 rpm in a microcentrifuge for 2-3 seconds to pellet the cells. The supernatant was poured into the waste container and the pellet resuspended in the residual
- 25 broth by pipetting up and down. This material was plated (approximately 100 µl) onto LB/Agar-Amp (100 µg/ml) plates and labeled as 10x. All plates were inverted in a 37°C incubator overnight.

3.8 Transformant Screening

Resulting bacterial colonies were patch-plated onto fresh LB/Agar-Amp (100 µg/ml) and placed inverted in a 37°C incubator overnight. The following day, 50 µl of RODI-water was aliquoted into 0.5 ml tubes. Using a sterile plastic pick, a small amount of each patch was scraped into one of the 0.5 ml tubes containing water. These were then placed at 95°C for 10 minutes, and spun at 14,000 rpm for 10 minutes in a microcentrifuge. To identify the proper insert in pYD1-5'3', colonies were screened by PCR using Taq Polymerase (Promega) and primers 4 and 10.

10 The PCR reaction was performed using the following master mix.

Component	4 rxs	10 rxns	15 rxns	20 rxns	25 rxns	30 rxns
RODI-water	161.5 µl	355.5 µl	516.8 µl	678.3 µl	839.8 µl	1001.3 µl
H ₂ O	25 µl	55 µl	80 µl	105 µl	130 µl	155 µl
MgCl ₂	10 µl	22 µl	32 µl	42 µl	52 µl	62 µl
15 P1 (100 µM)	1 µl	2.2 µl	3.2 µl	4.2 µl	5.2 µl	6.2 µl
P2 (100 µM)	1 µl	2.2 µl	3.2 µl	4.2 µl	5.2 µl	6.2 µl
dNTPs (10 mM)	40 µl	88 µl	128 µl	168 µl	208 µl	248 µl
Taq polymerase	1.5 µl	3.3 µl	4.8 µl	6.3 µl	7.8 µl	9.3 µl

20 Forty eight µl of each master mix was added to a 0.5 ml tube along with 5 µl of the template prepared from the colony patches. Additionally, a tube using RODI-water as a template was also made as a negative control. These tubes were then placed in the Ericomp Delta Cycler and cycled as follows: 96°C, 4 minutes; 30x (94°C, 30 seconds; 50°C, 1

25 minute; 72°C, 1 minute 25 seconds), 72°C, 4 minutes; 4°C, Hold. The PCR products were then run on 1.5% Agarose/TAE gels to check for positive colonies.

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3.9 Glycerol Stocks

One hundred twenty ml of LB-Amp (100 µg/ml) was then inoculated from a patch pYD1-5'3'/2 and shaken at 225 rpm overnight at 37°C. Two x 1 ml of this culture was then spun at 14 Krpm for 2-3
5 seconds to pellet the cells. These were resuspended in LB-Glycerol (30%) and frozen at -80°C.

3.10 MIDI Plasmid Preparation

Qiagen Midi-Prep was performed on the remaining culture using
10 the following modified protocol.

1. Spin 150 ml of each culture at 7 Krpm in GSA rotor for 10 minutes to pellet.
2. Decant Supernatant
3. Resuspend pellet in 4 ml P1 buffer; Transfer to 50 ml Falcon tube.
- 15 4. Rinse centrifuge bottle with 1 ml P1 buffer and transfer to the Falcon tube.
5. Add 5 ml P2 buffer; invert gently; incubate 5 minutes at room temperature or until lysed (no more than 12 minutes).
6. Add 5 ml P3 buffer; mix as above; incubate 10 minutes on ice.
- 20 7. Transfer supernatant to Qiagen Syringe Filter; Let sit for 10 minutes.
8. Equilibrate Q-100 tip with 4 ml QBT.
9. Gently push plunger to filter supernatant onto Q-100 tip.
10. Allow to drain by gravity.
11. Wash with 10 ml 2x QC.

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12. Elute into 50 ml polypropylene centrifuge tube with 5 ml QF.
13. Add 14 ml 100% EtOH (or 8 ml isopropanol); Place in dry ice/ethanol bath for 10 minutes (or at -20°C for 2 hours or at 4°C overnight).
14. Spin at 15 K x G for 30 minutes at 4°C.
15. Wash with 5 ml 70% ethanol; Spin 15 K x G for 20 minutes.
16. Air Dry.
17. Resuspend in 150 µl EB.

DNA concentration was measured as before.

Sample	A280	A260	280/260	260/280	Concentration
pYD1-5'3'/2	0.1708	0.3010	0.5675	1.7621	602 µg/ml

3.11 In Vitro Ligation to Create Full-length cDNA Chimeric Template and RNA Production Digestion with AatII and BstBI

The plasmid and fragment H were then digested with Aat II and BstB I (NEB) in a sequential digest as follows.

15 pYD1-5'3'/2 (AatII digest)

pYD1-5'3'/2 (10 µg)	16.6 µl
Buffer 4 (NEB)	5 µl
Aat II (NEB)	4 µl
H ₂ O	24.4 µl
Volume	50 µl

Fragment H (AatII digest)

Fragment H (0.7 µg)	51.0 µl
Buffer 6 (NEB)	5 µl
Aat II (NEB)	3 µl
H ₂ O	0 µl
Volume	60 µl

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Both reactions were incubated in a 37°C block overnight. Five µl of each digestion was run out on a 1.5% Agarose/TAE gel to check for complete digestion. The digestion was then incubated at 65°C for 20 minutes to inactivate the enzyme. 2.5 µl Bst BI (NEB) was added to each reaction and placed at 65°C overnight. The expected results of the digest are as follows:

	pYD1-5'3'/2	Fragment H
	5.6 kb	7.2 kb
10	0.14 kb	0.1 kb

The largest band from each reaction was gel excised and the UV concentration was determined (as previously described).

	Sample	A280	A260	280/260	260/280	Concentration
15	pYD1-5.2 fragment	0.0089	0.0154	0.5777	1.7309	30.8 ng/µl
	Fragment H	0.0020	0.0018	1.1333	0.8824	3.6 ng/µl

There was not enough fragment H for the ligation. Another 50 µl of fragment H was cleaned over a Qiagen Qiaquick column and digested with Aat II and Bst BI as described previously. The digested fragment was then gel excised as before and the UV concentration determined.

	Sample	A280	A260	280/260	260/280	Concentration
25	Fragment H	0.0000	0.0021	0.0000	NA	4.2 ng/µl

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3.12 Precipitation of Fragments

The following was prepared in a 1.5 ml tube.

5	pYD1-5'3'/2 fragment (298 ng)	9.7 μ l
	Fragment H (3.6 ng/ μ l)	38 μ l
	Fragment H (4.2 ng/ μ l)	32 μ l
	Water	20.3 μ l
	Volume	100 μ l

The fragments were then ethanol precipitated and resuspended in
 10 43.5 μ l of water to facilitate the ligation reaction.

3.13 Ligation

The following ligation reaction was setup using high concentration
 T4 DNA ligase (NEB). The ligations were incubated at 16°C overnight.

15	Precipitated fragments	43.5 μ l
	100x BSA (NEB)	0.5 μ l
	T4 DNA ligase buffer (NEB)	5 μ l
	*T4 DNA ligase	1 μ l
	Volume	50 μ l

20 * heat reaction at 37° for 5 minutes, then
 briefly chill on ice before adding ligase

3.14 Linearization of Template

The ligation was heat inactivated at 65°C for 10 minutes. The
 25 ligated material was then linearized at the 3' end of the Yellow Fever
 sequence to allow proper RNA transcription. 5.5 μ l of Buffer 2 (NEB)
 was added to the ligation, followed by 1.5 μ l XhoI (NEB), and then the
 reaction was put at 37°C for 2 hours.

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3.15 Linear cDNA extraction (RNase free phase)

1. Add H₂O to 100 µl total volume.
2. Add 1/10th volume 3 M Sodium Acetate
3. Add 100 µl Phenol/Chloroform/Isoamyl Alcohol and spin at 14 Krpm .
- 5 for 5 minutes in a microcentrifuge. Extract upper layer into RNase-free 1.5 ml tube. Repeat once.
4. Add 100 µl RNase-free Chloroform. Spin at 14 Krpm for 5 minutes in a microcentrifuge. Extract upper layer into RNase-free 1.5 ml tube. Repeat once.
- 10 5. Add 200 µl 100% RNase-free ethanol.
6. Place on dry-ice/ethanol bath for 10 minutes.
7. Spin at 14 Krpm for 20 minutes in a microcentrifuge.
8. Wash with 200 µl 70% ethanol (RNase-free).
9. Repeat 70% ethanol wash two more times.
- 15 10. Dry in Speed-Vac for 8 minutes (or until no more ethanol is present).
11. Resuspend in 22 µl nuclease free water from the SP6 kit listed below.

3.16 SP6 transcription

- The following reaction was setup using the SP6 transcriptase kit
- 20 (Epicentre) and Rnasin (Promega) in an RNase-free 1.5 ml tube using RNase-free tips in a BL-2 hood. The reaction was then placed in a 40°C water bath for 1 hour.

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5.	Capping NTP solution	6 μ l
	10x buffer	2 μ l
	20 mM Cap Analog	3 μ l
	100 mM DTT	2 μ l
	Linearized DNA	5 μ l
	Rnasin	0.5 μ l
	SP6 transcriptase	2 μ l
	Volume	20.5 μ l

10

3.17 Transfection with RNA

Three six well tissue culture plates were seeded with Vero-PM cells (p#162 from Cell Culture Facility) (2 plates at 1×10^6 cells/well and 1 plate at 2×10^6 cells/well) in growth media (Minimum Essential Media, Sodium Pyruvate, Non-Essential Amino Acids, Penicillin/Streptomycin, and 5% Fetal Bovine Serum) and placed in a 37°C CO₂ incubator until confluent.

The following transfection reactions were made using Lipofectin (Life Technologies) and RNase-free PBS (Sigma).

20 YF/DEN-1

PBS	250 μ l
Lipofectin	20 μ l
YF/DEN-1 RNA	10 μ l
Volume	280 μ l

25

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Total RNA control

PBS	250 μ l
Lipofectin	20 μ l
YF/JE total RNA	10 μ l
5 Volume	280 μ l

Lipofectin control

PBS	260 μ l
Lipofectin	20 μ l
10 Volume	280 μ l

1. Allow reactions to sit at room temperature for 10 minutes, and then remove Media from the six well plates.
2. Wash 3 times with PBS.
- 15 3. Remove last of PBS.
4. Overlay with each lipofectin reaction (add the YF/DEN-1 RNA to the 2×10^6 cells/well plate). Add 280 μ l media to the remaining wells.
5. Rock for 10 minutes at room temperature.
6. Wash 2 times with media.
- 20 7. Add 2 ml of media to each well and place in the 37°C CO₂ incubator for 4 days or more.

3.18 Harvest of the first Vero-PM passage (P1)

- The supernatant from YF/DEN-1 was harvested on day 6 by
- 25 splitting the 2 ml of supernatant between two cryovials (each containing 1

-80-

ml FBS) that were labeled YF/DEN-1 (PCR) (P1). The cell monolayer was harvested with 1 ml Trizol into a 1.5 ml tube. All vials and tubes were then placed at -80°C.

5 3.19 Amplification of YF/DEN-1

Vero-PM Passage #2

1. Three T-25 flasks containing Vero-PM cells (p#166) were obtained from the Cell Culture Facility. A frozen aliquot of YF/DEN-1 (PCR) (P1) was removed from the
- 10 -80°C freezer, thawed, and then placed on ice. The same was done for an aliquot of YF/JE (frozen stock from the P1 control transfection) to be used as a control.
2. Media was removed from each T-25 flask.
3. One ml of YF/DEN-1 (PCR) (P1) was added to the first flask, 1 ml of
- 15 media only (the same as was used in the transfection) was added to the second flask, and 1 ml of YF/JE(P1) was added to the third flask.
4. Each flask was then put in the 37°C, 5% CO₂ incubator for 1 hour with rocking every 10 minutes.
5. Four ml of media was then added to each flask.
- 20 6. The flasks were then placed in the 37°C, 5% CO₂ incubator for 4 days.

Harvest P2 and Passage #3

1. The supernatant from YF/DEN-1 flask was harvested on day 7.
2. Five hundred ml of YF/DEN-1(P2) was harvested and subsequently overlaid onto a T-25 flask containing Vero-PM cells (p#168 from the
- 25 Cell Culture Facility).

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3. Five hundred ml of media (same as used for transfection) was added to the monolayer.
4. One ml of media only was added to a control flask.
5. The flasks were placed in a 37°C CO₂ incubator and rocked every 15 minutes for 1 hour.
6. Meanwhile, the remaining YF/DEN-1(P2) was harvested into 4 cryovials containing 1 ml FBS and 1 cryovial containing 0.5 ml FBS and labeled as YF/DEN-1(P2). The cell monolayer was harvested with 3 ml Trizol into 1.5 ml tubes. All vials were placed at -80°C in a box labeled
10 YF/DEN-1.
7. After infection (Step 5), 4 ml of media was added to each flask and were transferred to the incubator for 4 or more days.

Harvest of P3

- The supernatant from YF/DEN-(P3) was harvested on day 5 by
15 splitting the 5 ml of supernatant between five cryovials (each containing 1 ml FBS), which were labeled YF/DEN-1(P3). The cell monolayer was harvested with 3 ml Trizol into 1.5 ml tubes. All vials and tubes were then placed at -80°C.

20 3.20 Virus Identification

The RNA from P3 was extracted using Trizol methods according to the manufacturer's protocol, RT-PCR was performed followed by sequencing of the YF/DEN-1 prME region 5', 3' junctions, inclusive. The expected sequence of the prME region was confirmed.

4.0 Construction of ChimeriVaxTM YF/DEN3

A viable yellow fever/dengue type 3 chimera (YF/DEN3) was constructed that contains the pre-membrane (prM) and envelope (E) genes of dengue type 3 virus (DEN3) replacing the corresponding prM-E region of the genome of the attenuated 17D yellow fever virus (YF). Virion RNA of wild-type DEN3 (strain PaH881/88) was used as a template to synthesize by RT-PCR two cDNA fragments that cover the DEN3 prM-E region. These fragments were cloned and sequenced. A modified protocol was used to prepare infectious YF/DEN3 *in vitro* RNA transcripts in which three appropriate DNA fragments were ligated *in vitro* followed by linearization with XhoI and *in vitro* transcription with SP6 RNA polymerase (standard ChimeriVax protocol employs two-fragment ligation). Following transfection of Vero PM cells with the RNA transcripts, virus-specific CPE was detectable as early as on day 5 post-transfection (and on day 3 postinfection in subsequent passages). The presence of the chimeric virus in the post-transfection (postinfection) media and the DEN3-specificity of its prM-E region were confirmed by RT-PCR and sequencing.

These results demonstrate that a chimeric YF virus containing DEN3-specific envelope is readily recoverable. The obtained YF/DEN3 chimera is a candidate part of our proposed tetravalent vaccine directed against all four dengue virus serotypes (dengue types 1-4).

The purpose of these experiments was to determine whether it is possible to create a viable YF/DEN3 chimera containing DEN3-specific envelope. At the same time, the proposed chimera was designed to contain the prM-E region from a pathogenic wild type strain of DEN3 (a prerequisite for high immunogenicity) in a backbone of the 17D vaccine

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strain of YF that includes the 5' and 3' UTRs, the C gene, and the nonstructural protein genes, NS1-5, (a prerequisite for safety).

To engineer a YF/DEN3 chimera containing the prM-E cassette from DEN3 in place of the prM-E cassette of YF we first wanted to use
5 the two-plasmid approach that was successful in previous studies where 17D YF virus (Rice *et al.*, New Biol. 1:285-296, 1989) and the YF/JE chimera (Chambers *et al.*, J. Virol. 73:3095-3101, 1999) were recovered following *in vitro* transcription and transfection. The DEN3 (strain PaH881/88) prM-E region was RT-PCR amplified in two adjacent
10 fragments (Fig. 29). To determine consensus sequence of this region of the parental virus, the RT-PCT fragments were directly sequenced in both directions. Since oligonucleotide primers used to synthesize these fragments were designed based on the published sequence of the H87 reference strain of DEN3 (Osatomi *et al.*, Virology 176:643-647, 1990),
15 actual viral sequences in the primer areas (at the beginning of prM, nucleotides 437-459; at the junction between the two fragments, nucleotides 1079-1131; and at the end of E, nucleotides 2385-2413) could not be determined. A total of 83 nucleotides changes were found compared to H87 strain. The rate of nucleotides differences, 4.44%, was
20 similar to that (4.5%) reported previously by Delenda *et al.* (J. Gen. Virol. 75:1569-1578, 1994) who sequenced roughly 80% of PaH881/88 E gene. Although the majority of nucleotides differences in the 80% E area coincided with those found by Delenda *et al.* (V. Deubel, personal communication) (53 changes coincided), there were 4 additional changes
25 that were not found by Delenda *et al.* In addition, we did not observe 3 of the changes reported by these authors. The PaH881/88 virus (a starting material in our experiments) was isolated from a patient by single amplification in mosquito AP61 cells. We propagated this virus in C6/36,

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another mosquito cell line. Sequences of both our P1 and P3 viruses were found to be identical indicating that there was no selective pressure on the virus in C6/36 cells. Therefore, it is more likely that the few discrepancies were due to sequencing mistakes in (Delenda *et al.*, J. Gen. Virol. 75:1569-1578, 1994). They resulted in three discrepancies in the amino acid sequence of the corresponding region of E protein (C-terminally truncated E; compare Table 32 below with Table 1 in Delenda *et al.*, J. Gen. Virol. 75:1569-1578, 1994). A complete list of amino acid differences we found in the prM-E region between the H87 and PaH881/88 strains is given in Table 33 (a total of 12 changes).

The RT-PCR fragments were used to replace corresponding JE-specific sequences in YFM5'3'IV JE SA14-14-2 and YFM5.2 JE SA 14-14-2 plasmids, which resulted in 5'3'/Den3 and 5.2/Den3 plasmids (Fig. 30). Inserts of both plasmids were sequenced. Since an extra XhoI site was found in the DEN3-specific region of 5'3'/Den3, the site was ablated by silent mutagenesis that resulted in 5'3'/Den3/DXho plasmid. The insert of this plasmid was also sequenced to ensure the absence of mutations introduced by PCR.

Difficulties were encountered in obtaining high quality 5.2/Den3 plasmid without mutations within its DEN3-specific region. Different growth conditions of plasmid-containing cultures and modifications in the extraction protocol (*e.g.*, reduction of alkali concentration in the lysis buffer), as well as growth of the plasmid in different *E. coli* strains were examined to overcome these difficulties. Finally, a clone of the plasmid (#26) was selected (propagated in ABLE C cells) that was of high quality and contained no nucleotide changes except for a single one nucleotide deletion at the 3' end of the DEN3 insert which could be easily eliminated

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by PCR (other sequenced clones contained more mutations). Therefore, the standard ChimeriVax procedure for preparation of infectious *in vitro* RNA transcripts that employs two fragment ligation prior to *in vitro* transcription was modified. According to the standard protocol, the large

5 BstBI-AatII fragment from 5.2/Den3 would be ligated with the large BstBI-AatII fragment of 5'3'/Den3/DXho (see in Fig. 30). Instead, to correct the deletion, three-fragment ligation was done (Fig. 30). The DEN-3 part of 5.2/Den3 was PCR-amplified on the #26 clone template with high-fidelity LA Taq polymerase and digested with BstBI and EheI

10 (isoschizomer of NarI). The opposite PCR primer was expected to correct the deletion. Second fragment, corresponding to the NarI-AatII part of 5.2/Den3, was derived by digestion of YFM5.2 JE SA14-14-2 with EheI and AatII. The two fragments were ligated with the large BstBI-AatII fragment of 5'3'/Den3/ Δ Xho. Ligation products were digested with XhoI

15 and transcribed *in vitro* with SP6 RNA polymerase.

Vero PM cells (at passage 149) grown in 6 well plates were transfected with the *in vitro* RNA transcripts. A first indication that the expected YF/DEN3 chimera was present was the appearance of CPE characteristic of other chimeras created to date based on the YF backbone.

20 It was first noticeable on day 5 post-transfection and became apparent (~10% of detached and rounded cells) on day 7 when virus-containing medium was harvested (P1). Subsequent P2 and P3 viruses were obtained by infecting fresh monolayers of Vero PM cells (at passages 150 and 151, respectively) with the P1 and P2 viruses (1 and 0.5 ml of the viruses were

25 used for each infection, respectively) and harvesting the virus when apparent CPE (~10%) was observable (on days 3 and 4 for P2, and day 3 for P3).

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The presence and DEN3-specificity of the YF/DEN3 chimera was confirmed by RT-PCR with YF- and/or DEN3-specific primers using P1 and P2 virion and intracellular RNAs as templates. All these reactions yielded specific RT-PCR products of expected sizes. As was expected, when DEN3-specific primers were used for RT-PCR on a control YF/JE RNA template, no product was recovered. The entire prM-E region of the P2 virus was sequenced. This also confirmed that the chimera contained the DEN3 envelope glycoprotein genes. In addition to the silent mutation introduced to ablate the XhoI site in the E gene, only one more silent nucleotide change was detected in the virus (A→G at nucleotide 2341 in the chimeric genome that corresponds to nucleotide 2296 in the parental DEN3). Since one of the DNA fragments used in the three-fragment ligation was synthesized by PCR (albeit on a plasmid template with known sequence), it is possible that the P1-3 viruses described here contain minor subpopulations with mutations introduced by PCR. To ensure homogeneity, these viruses can be plaque-purified and then sequenced. In addition, we are currently developing alternative cloning techniques that, if necessary, will allow recreation of the YF/DEN3 genome without using the intermediate PCR step. For instance, the DEN3-specific BstBI-NarI fragment of 5.2/Den3 plasmid was recently cloned without any mutations in low-copy number vectors (pCL and pACYC series). This fragment can be excised from the new plasmids and used instead of the PCR fragment in the three-fragment ligation to regenerate the chimera.

In conclusion, the prM-E region of the PaH881/88 DEN3 was sequenced and cloned. We demonstrate that a recombinant flavivirus genome (*e.g.*, YF/DEN3 in this study) can be reconstituted *in vitro* by using three-fragment ligation (instead of two-fragment ligation used previously to create other YF chimeras). This approach can be helpful in

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overcoming technical difficulties that are often encountered during cloning of genetic material from many flaviviruses in *E. coli* (especially dengue viruses). A viable 17D YF/DEN3 chimeric virus was recovered which is yet another successful example of the usefulness of the approach

- 5 developed by Chambers *et al.* (Chambers *et al.*, J. Virol. 73:3095-3101, 1999; see above), in which the prM-E cassette of a heterologous flavivirus is inserted into the YF backbone such that the hydrophobic signal for prM remains YF-specific.

The materials and methods used to make and characterize the
10 YF/DEN3 chimera are described as follows.

4.1 Virus and cells

DEN3 strain PaH881/88 was isolated from a patient by single amplification in AP61 (mosquito) cells. C6/36 cells were maintained in
15 MEM (Gibco, Cat.# 11095-072) supplemented with 10% FBS (HyClone, Cat.# SH30070103) and 1x non-essential amino acids (Sigma, Cat.# M7145) (OraVax ML-8 medium, Lot.# 108H2308) at 28°C under 5% CO₂. DEN3 was passaged two times by infecting monolayers of C6/36 at an unknown MOI and harvesting virus-containing growth media on day 7
20 post-infection (P1 and P2) and one time by infecting C6/36 cells with the P2 virus at an MOI of ~ 0.01 pfu/cell and harvesting the medium on day 6 (P3; pronounced virus-specific CPE was observed in P3).

Virus-containing media were mixed with an equal volume of FBS, aliquoted and stored at 70°C. Following transfection/infection, Vero PM
25 cells were maintained in MEM (Gibco, Cat.# 11095-080, Lot.# 1017611) supplemented with 5% heat-deactivated FBS (OraVax Lot.# AGE6578)

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and penicillin/streptomycin (100 U/0.1 mg per ml; Sigma, Cat.# P-0781, Lot.# 78H2386) at 37°C under 5% CO₂.

4.2 RNA extraction

5 DEN3 virion RNA was extracted from 0.5 ml of clarified P3 virus-containing medium using TRI Reagent-LS (Molecular Research Center, Inc., Cat.# TS-120) according to the manufacturer's procedure and redissolved in 10 µl H₂O. Alternatively, (*e.g.*, to confirm the presence of YF/DEN3 chimera), intracellular RNA from infected cells was extracted
10 using TRI Reagent (Molecular Research Center, Cat.# TR-118).

4.4 Reverse transcription, PCR, and sequencing

First strand cDNA syntheses were done in a total volume of 20 µl using 2.5 µl of DEN3 virion RNA as a template, indicated oligonucleotide
15 primers (see below) and SuperScript II reverse transcriptase (Gibco, Cat.# 18064-014) according to the manufacturer's procedure. Prior to PCR, RT products were treated with RNase H (Promega). High-fidelity PCR on RT products or indicated plasmid DNAs as templates was done using the GeneAmp XL PCR kit (Perkin Elmer, Cat.# N808-0192), or the TaKaRa
20 LA Taq polymerase kit (PanVera, Cat.# TAK RR002M) according to the protocols provided by the kit manufacturers with a GeneAmp 2400 thermocycler (Perkin Elmer). Sequencing of indicated PCR products or plasmid DNAs was done using the ABI Prism dRhodamine Terminator Cycle Sequencing kit (Perkin Elmer, Cat.# 403042). Sequencing reaction
25 products were resolved with ABI Prism 310 automated sequencer (Perkin Elmer). Data were analyzed using Sequencher 3.0 software and stored on the Internet

(ORAVAX/VOLTEMP/GROUPS/LABTECH/KOSTIA/folder "KP sequencing data"/Exp.##). With each area of interest, both DNA strands were sequenced and analyzed. Oligonucleotide primers are listed in Table 32.

5 Primers were ordered from Custom Primers (Life Technologies/GibcoBRL). In the names of primers, numbers indicate approximate localization of oligos on the DEN3 genome and "+/-" indicates orientation of each primer, with the following exceptions: oligo 5 is colinear with a region of YFM5'3' series of plasmids upstream from the
10 NotI cloning site; oligos 6 (opposite) and 7 (direct) are YF-specific; the former corresponds to the end of YF C gene; oligos 15 (direct) and 16 (opposite) were designed for amplification and sequencing of inserts in the YFM5.2 series of plasmids and correspond to regions of the plasmids located within ~ 60 nucleotides upstream and downstream from the
15 corresponding inserts, respectively; oligo 8 (direct) was used to mutate the XhoI site at nucleotide 1052 of the recombinant YF/DEN3 genome (within 5'3'/Den3 plasmid); and oligo 17 is colinear with the SP6 promoter and a few of the 5' terminal nucleotides from YF.

20 4.3 DNA manipulations

Standard molecular biology techniques were in accordance with Maniatis *et al.*, Molecular Cloning: a Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1992. All restriction enzymes, except for EheI (Fermentas) and T4 DNA ligase,
25 were from New England Biolabs.

4.4 Construction of 5'3'/Den3/DXho plasmid

The 5' terminal part of the DEN3 prM-E region was synthesized on purified virion RNA of the P3 virus by RT-high-fidelity PCR (XL PCR) using oligonucleotide primers 1 and 2. It starts precisely at the beginning
5 of the coding region for prM protein (at nucleotide 437; DEN3 nucleotide numbering is according to the sequence of H87 reference strain (Osatomi *et al.*, Virology 176:643-647, 1990)) that is generated by signalase and ends at nucleotide 1106 and thus contains the entire prM and approximately one-seventh of the E gene. In addition to DEN3 sequences,
10 the resulting RT-PCR product contains the last 23 nucleotides of the YF C gene for subsequent overlapping PCR (at its 5' end). The last six nucleotides of DEN3 sequence (nucleotides 1101-1106) are changed to a BstBI site by introduction of three silent nucleotide changes for subsequent *in vitro* ligation, which is followed by a NheI site for cloning.

15 To combine the RT-PCR product with the upstream YF-specific sequences, a fragment of YFM5'3'IV JE SA14-14-2 plasmid (an analog of 5'3'/Den3 plasmid used to generate a similar YF/JE chimera (Chambers *et al.*, J. Virol. 73:3095-3101, 1999; see above)) containing SP6 RNA polymerase promoter followed by the 17D YF 5' UTR and C gene (first
20 481 nucleotides of YF genome) was amplified by XL PCR with oligos 5 and 6. For overlapping PCR, the resulting DNA fragment was mixed with the RT-PCR product and XL PCR amplified with oligos 2 and 5. Consensus sequence of the dengue type 3 region was determined by sequencing the RT-PCR and the overlapping PCR products in both
25 directions using oligos 1, 2, 5, 7, 9, and 10.

The overlapping PCR product was used to replace the short NotI-NheI fragment in YFM5'3'IV JE SA14-14-2. The replaced region of

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the resulting 5'3'/Den3, which is a pBR322-based plasmid maintained in *E. coli* MC1061RecA- cells, was sequenced using oligos 1, 2, 9, 10, and 17, and a correct clone (#3) was selected, which does not have any mutations compared to the consensus sequence.

- 5 Sequencing revealed that the DEN3-specific portion of 5'3'/Den3 contains an additional XhoI site located in the beginning of E gene (nucleotides 1007-1012 in DEN3 genome). Another XhoI site used for linearization prior to *in vitro* transcription (see below) is located at the end of YF sequence in 5'3'/Den3. The additional site was destroyed by silent
10 oligonucleotide-directed mutagenesis (LA PCR; DEN3-specific C at nucleotide 1009 was changed to G) using oligo 8, resulting in a plasmid 5'3'/Den3/DXho. The entire region of the plasmid replaced during mutagenesis was sequenced with oligos 1, 2, 9, 10, and 17 and a clone (#10) was selected that does not have any mutations, except for the desired
15 C to G nucleotide change.

4.5 Construction of 5.2/Den3 plasmid

- The 3' terminal part of DEN3 prM-E region was RT-PCR amplified (XL PCR) on the P3 virion RNA template using primers 3 and 4. It starts
20 with BstBI site introduced at nucleotides 1101-1106 for in-frame ligation with 5'3'/Den3/DXho plasmid and ends with a NarI site introduced precisely at the 3' end of E gene (nucleotides 2408-2413) for in-frame ligation with YF NS1. The NarI site that leads to Q to G change of the penultimate amino acid residue in the DEN3 E was used previously to
25 generate YF/JE chimera (Chambers *et al.*, J. Virol. 73:3095-3101, 1999; see above). An NheI cloning site was placed upstream from the BstBI site. The consensus sequence of this DEN3 region was determined by

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sequencing of the RT-PCR product in both orientations using oligos 3, 4, 11, 12, 13, and 14.

The PCR product was cloned in place of the short NheI-NarI fragment in YFM5.2 JE SA14-14-2 plasmid (Chambers *et al.*, J. Virol. 73:3095-3101, 1999; see above), resulting in the 5.2/Den3 plasmid. Originally it was propagated in *E. coli* MC1061RecA- cells, and the DNA extracted from these cells was of poor quality (partially denatured), which hindered restriction digestions. Subsequently, several of the extracted plasmid clones were propagated in *E. coli* ABLE C cells (Stratagene), and good quality DNAs were recovered. Six of these clones were thoroughly analyzed by restriction analysis and then sequenced. For sequencing, the DEN3-specific insert with adjacent regions of the vector was PCR amplified using oligos 15 and 16, and the product was sequenced in both orientations with oligos 11, 12, 13, 14, 15, and 16. Clone #26 was chosen for subsequent manipulations. It contains no mutations, compared to the consensus sequence, except for a single nucleotide deletion (nucleotide 2407, in the region of the opposite PCR primer 4).

4.6 *In vitro* transcription and transfection

These techniques were essentially as described by Rice *et al.* (New Biol. 1:285-296, 1989). Approximately 1 µg total of equimolar amounts of appropriate gel-purified DNA fragments were ligated overnight in 20 µl volume at 40°C. T4 DNA ligase was heat-inactivated (10 minutes, 65°C). Ligation products were digested with XhoI to provide run-off transcription, phenol-chloroform extracted, and precipitated with ethanol. The resulting DNA was transcribed *in vitro* with SP6 RNA polymerase in the presence of m7G(5')ppp(5')G cap analog and Rnasin in a 20 µl reaction (AmpliScribe SP6 Kit With Cap Analog; Epicentre Technologies; Cat.#

AS2606C2). RNA transcripts were analyzed by electrophoresis of 2 μ l aliquots in 1% agarose gel. Monolayers of Vero PM cells grown in 6 well tissue culture plates were transfected with RNA transcripts using Lipofectin reagent (Gibco, Cat.# 18292-011). Following transfections, 5 cells were incubated as is described above, and virus-containing media were harvested on indicated days post-transfection, mixed with equal volume of FBS, aliquoted and stored at -70°C.

5.0 Construction of ChimeriVaxTM YF/DEN-4

10 The purpose was to generate yellow fever/dengue 4 (YF/DEN-4) chimeric virus as a dengue vaccine candidate (see Figs. 31 and 32). To attain this, we used a technology derived from the construction of Yellow fever/ Japanese encephalitis (YF/JE SA 14-14-2) chimeric virus (Chambers *et al.*, J. Virol. 73:3095-3101, 1999). It consists of a two 15 plasmid system which originally encoded the yellow fever (YF) genome. These YF plasmids were created by Charlie Rice (Rice *et al.*, New Biol. 1:285-296, 1989). The structural membrane precursor and envelope protein genes, *i.e.*, the prME portion, of the YF genome plasmids with that of JE SA14-14-2 sequence and used the resulting plasmids to produce 20 RNA *in vitro*, which was then transfected into cells to produce live YF/JE chimeric virus. The system seemed suitable to construct other flavivirus chimeras using YF as backbone and here we describe the use of dengue 4 as a start point. The dengue 4 strain, #1228 isolated in 1978 in Indonesia and passaged twice in Mosquitoes, was passed once in C6/36 and total 25 RNA was isolated to synthesize cDNA for PCR of the prME region as needed for cloning. Here we describe in detail the procedures for construction of the YF/DEN-4 chimera. The dengue 4 prME region was

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first amplified and sequenced using primers derived from consensus sequences (Genbank). The sequence data created was applied to primer design which were used, with the cDNA produced earlier, as PCR starting point for assembly of the two-plasmid system for dengue 4 (*i.e.*, by replacing the corresponding prME JE sequences in each plasmid). A PCR product encoding dengue prM and 5' end of E was used as template, along with template encoding the capsid (C) gene of yellow fever derived from the plasmid pYF5'3'IV/JE SA 14-14-2, in an overlap extension PCR to result in a single fusion product which was then cloned into a fragment of pYFM5'3'IV where JE sequences were deleted. The 3' end of dengue 4 envelope protein gene was also amplified and then cloned into a vector fragment of pYFM5.2/JE SA 14-14-2, resulting in replacement of JE sequence with that of dengue 4. Both plasmids were then transformed into *E. coli* strain MC1061 (RecA-) and midi-scale plasmid cultures were grown. *In vitro* ligation of the two plasmids resulted in full-length virus DNA template of YF/DEN-4 for RNA transcription. All steps involving cDNA fragments and plasmids were carried out in a BL-2 lab designated for recombinant DNA work. Steps involving manipulations of infectious RNA and virus were carried out in a limited access BL-2+ virus lab.

20

5.1 Amplification of Dengue 4 Sequence

Dengue 4 cDNA was synthesized from RNA using the Superscript II™ method. All primers for this experiment were synthesized by Life Technologies and are listed in Table 34. Upon arrival as lyophilized material, primers were dissolved to 250 mM stock solutions in RODI-water. From this, 25 mM working solutions were made. The fragment encoding the SP6 promoter and yellow fever capsid (Fragment

25

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A) was amplified using the XL-PCR Reaction Kit TM (Perkin-Elmer Part# N808-0192), 0.5 ml (250 ng) of template pYF5'3'IV plus 3.5 ml RODI-water, and primers 1 and 2. The fragment encoding dengue 4 prM and the 5' end of E (Fragment B) was amplified using the XL-PCR

- 5 Reaction Kit TM (Perkin-Elmer Part#N808-0192) and primers 3 and 4. The fragment encoding the 3' end of dengue 4 envelope (Fragment C) was amplified using the same protocol but using primers 5 and 6. Each PCR reaction was performed as indicated in master mixes (see section 3.1, above).

- 10 For each reaction, the lower mix (LM) was added to a Perkin-Elmer thin-walled 0.2 ml tube. Next, Ampliwax 100 (Perkin-Elmer) was added to the tube, which was then placed in a Perkin-Elmer 2400 Thermal Cycler and heated to 80°C for 5 minutes, and then cooled to 4°C. The cDNA and UM were then added to the top of the wax layer. The tube was then
- 15 cycled in a Perkin-Elmer 2400 as follows: 94°C, 1 minute; repeat 30 x (94°C, 15 seconds; 53°C, 15 seconds; 68°C, 3 minutes), 72°C, 4 minutes; 4°C, hold. The expected sizes of the PCR fragments for cloning were as follows:

Fragment	Approximate Size (kb)
A	0.940
B	0.650
C	1.300

- 20 Forty µl of each fragment was then separated on a 1% Agarose/TAE gel and purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704). Next, the concentration of the purified fragments was determined by UV absorption using 1:40 dilutions in RODI-water.

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Sample	A280	A260	280/260	260/280	Concentration
Fragment A	0.0247	0.0340	0.7271	1.3754	68 ng/ μ l
Fragment B	0.0086	0.0152	0.5643	1.7721	30 ng/ μ l
Fragment C	0.0099	0.0179	0.5536	1.8065	36 ng/ μ l

5

5.2 Recombinant PCR

To create a fusion between the yellow fever capsid gene and the 5' end of the dengue 4 prM, a recombinant PCR technique,

Overlap-extension PCR was used to create Fragment E. The same basic

10 UM and LM were used and primers 1 and 4 replaced earlier primers; the cDNA templates used were mixes shown below:

	Fragment E	Fragment A control	Fragment B control
H ₂ O	37.8 μ l	39.1 μ l	38.7 μ l
Fragment A	0.9 μ l	0.9 μ l	0 μ l
15 Fragment B	1.3 μ l	0 μ l	1.3 μ l
Volume	40 μ l	40 μ l	40 μ l

The same protocol that was used for creation of Fragments A, B, and C was used except that only $\frac{1}{2}$ of the cDNA, UM, and LM were used

20 for the control reactions. The tube was then cycled in a Perkin-Elmer 2400 cyclor as follows: 94°C, 1 minute; repeat 30x (94°C, 15 seconds; 55°C, 15 seconds; 68°C, 2 minutes; 72°C, 7 minutes; 4°C, hold. The expected sizes were as follows.

Fragment	Approximate Size (kb)
25 E	1.59
A control	no product
B control	no product

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Forty μ l of Fragment E was then separated on a 1% Agarose/TAE gel and purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704). Next, the concentration of the purified fragment was determined by UV absorption using 1:40 dilutions in RODI-water.

5	Sample	A280	A260	280/260	260/280	Concentration
	Fragment E	0.0049	0.0110	0.4489	2.2276	22 ng/ μ l

5.3 Cloning of Fragments C and E into Yellow Fever Vectors

The fragments were then cloned into the yellow fever two-plasmid system by digestion of the purified Fragments C and E as well as the plasmids pYF5'3'TV and pYFM5.2/2 with appropriate restriction enzymes as shown below. The digested plasmids resulted in two bands. The smaller bands contain a fragment of Japanese encephalitis corresponding to either Fragment C or Fragment E for the new dengue 4 constructs. All restriction enzymes, buffers, and 100x BSA were from New England Biolabs. All the digestions were carried in a Perkin-Elmer 480 cyclor set to hold at 37°C overnight.

Fragment E digest

20	Fragment E (528 ng)	27 μ l
	NEB buffer 4	4 μ l
	10x BSA	4 μ l
	H ₂ O	3 μ l
	Not I	1 μ l
	Nhe I	1 μ l
25	Volume	40 μ l

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pYF5.2 digest

5	pYF5.2 (1 µg)	9.5 µl
	NEB buffer 2	2 µl
	10x BSA	2 µl
	H ₂ O	4 µl
	Sfo I	1.5 µl
	Nhe I	1 µl
	Volume	20 µl

10 pYFMIV5'3' digest

15	pYFMIV5'3' (1.02 µg)	2 µl
	NEB buffer 4	2 µl
	10x BSA	2 µl
	H ₂ O	12 µl
	Not I	1 µl
	Nhe I	1 µl
	Volume	20 µl

Fragment C digest

20	Fragment C (710 ng)	20 µl
	NEB buffer 2	4 µl
	10x BSA	4 µl
	H ₂ O	9.5 µl
	Sfo I	1.5 µl
	Nhe I	1 µl
	Volume	40 µl

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5.4 Vector Dephosphorylation

Calf Intestinal Phosphatase (CIP) from New England Biolabs (cat#290S) was diluted 1:10 in 1x CIP Buffer. One μ l of this dilution was then added to the pYFMIV5'3' digest. 0.62 μ l of stock CIP was added directly to the pYF5.2 digest. Both were incubated for 1 hour at 37°C. Then, 0.8 μ l 125 mM EDTA was added to the two tubes and placed at 75°C for 10 minutes to inactivate CIP

5.5 Gel Excision

10 The digested PCR fragments were separated on a 1.0% Agarose/TAE gel, while the digested plasmids were separated on a 0.8% Agarose/TAE gel. All were purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704).

15 5.6 Ligations

The digested Fragment E and pYF5'3'IV were ligated using T4 DNA Ligase (New England Biolabs cat#202S) to create pYD4-5'3'. The digested Fragment C and pYFM5.2 were ligated to create pYD4-5.2. All ligation reactions were incubated in a Perkin-Elmer 480 cyclor set to hold
20 at 16°C overnight.

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PYD4-5'3' Ligation

5	Fragment E (97.5 ng)	9.5 µl
	pYFM5'3' (50 ng)	3.6 µl
	H ₂ O	3.9 µl
	10x T4 ligase buffer	2 µl
	T4 DNA ligase	1 µl
	Volume	20 µl

PYD4-5.2 Ligation

10	Fragment C (56 ng)	4.5 µl
	pYFM5.2 (70 ng)	8.8 µl
	H ₂ O	3.7 µl
	10x T4 ligase buffer	2 µl
	T4 DNA ligase	1 µl
15	Volume	20 µl

pYFM-5'3' Control Ligation

20	Fragment E	0 µl
	pYFM5'3' (50 ng)	3.6 µl
	H ₂ O	13.4 µl
	10x T4 ligase buffer	2 µl
	T4 DNA ligase	1 µl
	Volume	20 µl

25

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pYF5.2 Control Ligation

Fragment C	0 μ l
pYFM5.2 (70 ng)	8.8 μ l
H ₂ O	8.2 μ l
5 10x T4 ligase buffer	2 μ l
T4 DNA ligase	1 μ l
Volume	20 μ l

5.7 Transformations

- 10 All four ligation reactions were transformed into *E. coli* strain MC1061 (recA-). An aliquot of MC1061 (OraVax Notebook 661-4) was removed from storage at -80°C and allowed to thaw on ice for one to two minutes. 0.9 ml of cold 0.1 M CaCl₂ was added to the cells. One hundred μ l of cells was aliquoted into five 12 ml culture tubes on ice. Ten μ l of
- 15 each ligation reaction was added to each culture tube, leaving the fifth tube as a negative (no DNA) control. Culture tubes were left on ice for 30 minutes. The tubes were heat shocked in a water bath at 42°C for 45 seconds. The tubes were put back on ice for 2 minutes. 0.9 ml SOC medium was added to each culture tube and incubated at 225 pm in a
- 20 shaking incubator at 37°C for 1 hour. Each transformation mix was aliquoted into 1.5 ml microcentrifuge tubes. One hundred μ l of each was spread onto LB/Agar-Amp (100 μ g/ml) plates and labeled as "neat." Each tube was spun at 14 Krpm in a microcentrifuge for 2-3 seconds to pellet the cells. The supernatant was poured into the waste container and the
- 25 pellet resuspended in the residual broth by pipetting up and down. This material was plated (approximately 100 μ l) onto LB/Agar-Amp (100 μ g/ml) plates and labeled as 10x. All plates were inverted in a 37°C incubator overnight.

5.8 Transformant Screening

- The resulting bacterial colonies were patch-plated onto fresh LB/Agar-Amp (100 µg/ml) and placed inverted in a 37°C incubator overnight. The following day, 50 µl of RODI-water was aliquoted into 0.5 ml tubes. Using a sterile plastic pick, a small amount of each patch was scraped into one of the 0.5 ml tubes containing water. These were then placed at 95°C for 10 minutes and spun at 14,000 rpm for 10 minutes in a microcentrifuge. To identify the proper insert in pYD4-5'3', colonies were screened by PCR using Taq Polymerase (Promega) and primers 4 and 7.
- 10 The pYD4-5.2 was screened using primers 5 and 6. The PCR reaction was performed using the following master mix.

	Component	4 rxns	10 rxns	15 rxns	20 rxns	25 rxns	30 rxns
	RODI-water	161.5 µl	355.5 µl	516.8 µl	678.3 µl	839.8 µl	1001.3 µl
	H ₂ O	25 µl	55 µl	80 µl	105 µl	130 µl	155 µl
15	MgCl ₂	10 µl	22 µl	32 µl	42 µl	52 µl	62 µl
	P1 (100 µM)	1 µl	2.2 µl	3.2 µl	4.2 µl	5.2 µl	6.2 µl
	P2 (100 µM)	1 µl	2.2 µl	3.2 µl	4.2 µl	5.2 µl	6.2 µl
	dNTPs (10 mM)	40 µl	88 µl	128 µl	168 µl	208 µl	248 µl
20	Taq Polymerase	1.5 µl	3.3 µl	4.8 µl	6.3 µl	7.8 µl	9.3 µl

- Forty eight µl of each master mix was added to a 0.5 ml tube along with 5 ml of the template prepared from the colony patches. Additionally, a tube using RODI-water as a template was also made as a negative control. These tubes were then placed in the Ericomp Delta Cyclor and
- 25 cycled as follows: 96°C, 4 minutes; 30x (94°C, 30 seconds; 50°C, 1 minute; 72°C, 1 minute 25 seconds), 72°C, 4 minutes; 4°C hold. The PCR products were then run on 1.5% Agarose/TAE gels to check for positive colonies.

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5.9 Glycerol Stocks

Five ml of LB-Amp (100 µg/ml) was then inoculated from a patch pYD4-5'3'/2 or pYD4-5.2/1 and shaken at 225 rpm overnight at 37°C.

One ml of this culture was then spun at 14 Krpm for 2-3 seconds to pellet
5 the cells. This was then resuspended in LB-Glycerol (30%) and frozen at -80°C.

5.10 MIDI Plasmid Preparation

Fifty µl of each glycerol stock was added to 150 ml LB-Amp (100
10 µg/ml) in separate 4 L flasks and shaken at 225 rpm overnight at 37°C.
Qiagen Midi-Prep (Qiagen) was performed using the following modified protocol.

1. Spin 150 ml of each culture at 7 Krpm in GSA rotor for 10 minutes to pellet.
- 15 2. Decant Supernatant.
3. Resuspend pellet in 4 ml P1 Buffer; transfer to 50 ml Falcon tube.
4. Rinse centrifuge bottle with 2 ml P1 buffer and transfer to the Falcon tube.
5. Add 6 ml P2 buffer; invert gently; 5 minutes at room temperature or
20 until lysed (no more than 12 minutes).
6. Add 6 ml P3; mix as above; 10 minutes on ice.
7. Transfer supernatant to Qiagen Syringe Filter; let sit for 10 minutes.
8. Equilibrate Q-100 tip with 4 ml QBT.
9. Gently push plunger to filter supernatant onto Q-100 tip.
- 25 10. Allow to drain by gravity.

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11. Wash with 2 x 10 ml QC.
12. Elute into 50 ml polypropylene centrifuge tube with 5 ml QF.
13. Add 14 ml 100% ETOH (or 8 ml isopropanol); Place in dry ice/ethanol bath for 10 minutes (or at -20°C for 2 hours or at 4°C overnight).
14. Spin at 15 K x G for 30 minutes at 4°C.
15. Wash with 5 ml 70% ethanol; Spin 15 K x G for 20 minutes.
16. Air Dry.
17. Resuspend in 150 µl EB.

10 DNA concentrations were measured as before.

Sample	A280	A260	280/260	260/280	Concentration
pYD4-5'3'/2	0.0516	0.0939	0.5498	1.1817	1.87.8 ng/µl
pYD4-5.2/1	0.0900	0.1407	0.6399	1.5629	281.4 ng/µl

15 *5.11 In Vitro Ligation to Create Full-length cDNA Chimeric Template and RNA Production*

Digestion with AatII and BstBI

Plasmids pYD4-5'3'/2 and pYD4-5.2/1 were digested with AatII and BstBI (NEB) in a sequential digest as follows.

20 YD4-5'3'/2 (AatII digest)

pYD4-5'3'/2 (8 µg)	42.5 µl
Buffer 4 (NEB)	5 µl
AatII (NEB)	2 µl
H ₂ O	0.5 µl
Volume	50 µl

25

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YD4-5.2/1 (AatII digest)

pYD4-5.2 (10 µg)	35.5 µl
Buffer 4 (NEB)	5 µl
AatII (NEB)	2 µl
H ₂ O	7.5 µl
Volume	50 µl

Both reactions were incubated in a 37°C block for 2 hours. Five µl of each digest was run out on a 1.5% Agarose/TAE gel to check for complete digestion. The pYD4-5'3'/2 digest did not cut completely so the reaction was cleaned over a Qiaprep spin column (Qiagen). The digest was repeated using this material and 3 µl of Aat II. In addition, 3 µl of Aat II was added to the existing pYD4-5.2/1 reaction. Both were incubated in a 37°C block, overnight. After confirmation of digest on another gel (as previously described), 2.5 µl Bst BI (NEB) was added to each reaction and placed at 65°C for 3 hours. The results of the digest were as follows.

PYD4-5'3'/2	PYD4-5.2/1
5.6 kb	7.2 kb
	2.0 kb
0.14 kb	0.4 kb

The largest band from each reaction was gel excised as and the UV concentration was determined (as previously described).

5.12 Ligation

The following ligation reaction was setup using high concentration T4 DNA ligase (NEB). The ligations were incubated at 16°C overnight.

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H ₂ O	13.5 µl
pYD4-5.2 fragment (600 ng)	23 µl
pYD4-5'3' fragment (300 ng)	7 µl
100x BSA (NEB)	0.5 µl
5 T2 DNA ligase buffer (NEB)	5 µl
*T4 DNA ligase	1 µl
Volume	50 µl

*Heat reaction at 37°C for 5 minutes, then
briefly chill on ice before adding ligase

10

5.13 Linearization of Template

The ligation was heat inactivated at 65°C for 10 minutes. The ligated material was then linearized at 3' end of the yellow fever sequence to allow proper RNA transcription, as follows: 5.5 ml Buffer 2 (NEB) was
15 added to the ligation, followed by 1.5 µl XhoI (NEB), and this reaction mixture was placed at 37°C for 2 hours.

5.14 Linear cDNA extraction (RNase free phase)

1. Add H₂O to 100 µl total volume.
- 20 2. Add 1/10th volume 3 M Sodium Acetate.
3. Add 100 µl Phenol/Chloroform/Isoamyl Alcohol and spin at 14 Krpm for 5 minutes in a microcentrifuge. Extract upper layer into RNase-free 1.5 ml tube. Repeat once.
4. Add 100 µl RNase-free Chloroform. Spin at 14 Krpm for 5 minutes in
25 a microcentrifuge. Extract upper layer into RNase-free 1.5 ml tube.
Repeat once.
5. Add 200 µl 100% RNASE-free ethanol.

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6. Place on dry-ice/ethanol bath for 10 minutes.
7. Spin at 14 K μ rpm for 20 minutes in a microcentrifuge.
8. Wash with 200 μ l 70% ethanol (RNase-free).
9. Repeat 70% ethanol wash two more times.
- 5 10. Dry in Speed-Vac for 8 minutes (or until no more ethanol is present).
11. Resuspend in 22 μ l nuclease free water from the SP6 kit listed below.

5.15 SP6 transcription

- The following reaction was setup using the SP6 transcriptase kit
- 10 (Epicentre) and Rnasin (Promega) in an RNase-free 1.5 ml tube using RNase-free tips in a BL-2 hood. The reaction was then placed in a 40°C water bath for 1 hour.

	Capping NTP solution	6 μ l
	10x buffer	2 μ l
15	20 mM Cap Analog	3 μ l
	100 mM DTT	2 μ l
	Linearized DNA	5 μ l
	Rnasin	0.5 μ l
	SP6 transcriptase	2 μ l
20	Volume	20.5 μ l

5.16 RNA Transfection

Two six well tissue culture plates were seeded with Vero-PM (p#153 OraVax notebook#743-7) cells at 7.4×10^5 cells/well in growth

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media (Gibco MEM; Sodium Pyruvate; NEAA; Penicillin/Streptomycin; 5% fetal bovine serum), and placed in a 37°C CO₂ incubator until confluent.

The following transfection reactions were made using Lipofectin (Life Technologies) and RNase-free PBS (Sigma), and allowed to sit at room temperature for 10 minutes.

YF/DEN-4

10	PBS	250 µl
	Lipofectin	20 µl
	YF/DEN-4 RNA	10 µl
	Volume	280 µl

Total RNA control

15	PBS	250 µl
	Lipofectin	20 µl
	YF/JE total RNA	10 µl
	Volume	280 µl

Lipofectin control

20	PBS	260 µl
	Lipofectin	20 µl
	Volume	280 µl

1. Remove Media from the 6 well plates.
- 25 2. Wash 3 times with PBS.
3. Remove last of PBS.
4. Overlay with each lipofectin reaction. Add 280 µl PBS for the remaining wells.

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5. Rock for 10 minutes at room temperature.
6. Wash 2 times with media (MEM, Sodium Pyruvate, NEAA, P/S, 5% FBS).
7. Add 2 ml of media to each well and place in the 37°C CO₂ incubator
- 5 for 4 days or more.

5.17 Chimeric Virus Harvest

The supernatant from YF/DEN-4 was harvested on day 6 by splitting the 2 ml of supernatant between two cryovials (each containing 1
10 ml FBS), which were labeled YF/DEN-4 (P1). The cell monolayer was harvested with 1 ml Trizol into a 1.5 ml tube. All vials and tubes were then placed at -80°C.

5.18 Amplification of YF/DEN-4

15 Passage #2

1. Three T-25 flasks containing Vero-PM cells (p#149) were obtained from the Cell Culture Facility. A frozen aliquot of YF/DEN-4 (P1) was removed from the -80°C freezer, thawed, then placed on ice. The same was done for an aliquot of YF/JE (frozen stock from the P1 control
20 transfection).
2. Media was removed from each T-25 flask.
3. Five hundred µl of YF/DEN-4(P1) was added to the first flask, 500 µl of media (MEalphaM, NEAA, Sodium Pyruvate, 5% FBS, P/S) was added to the second flask, and 500 µl of YF/JE(P1) was added to the third flask.

25

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4. Two hundred μ l of the media was added to each flask to ensure complete coverage.
5. Each flask was then put in the 37°C CO₂ incubator for 1 hour with rocking every 15 minutes.
- 5 6. 4.5 ml of media was then added to each flask.
7. The flasks were then placed in the 37°C CO₂ incubator for 4 days.

Harvest P2

The supernatant from YF/DEN-4 was harvested on day 4 by splitting the 5 ml of supernatant between five cryovials (each containing 1
10 ml FBS), which were labeled YF/DEN-4 (P2). The cell monolayer was harvested in 3 ml Trizol and aliquoted into 3 tubes. All vials and tubes were then placed at -80°C.

Passage#3 (Titration of P2)

1. A 12 well tissue culture plate containing Vero-PM cells (p#163) was
15 obtained from the Cell Culture Facility. A frozen aliquot of YF/DEN-4 (P2) was removed from the -80°C freezer, thawed, and then placed on ice.
2. Serial dilutions were made in a 24 well tissue culture plate ending at 10⁻⁴ in a total volume of 300 μ l. The plate was then placed on ice.
3. The media was removed from each well of the 12 well plate and 100 μ l
20 of virus stock, as well as each dilution, was added to the wells in duplicate. One hundred μ l of media only was added to the last set of wells as a negative control.
4. The plate was then put in the 37°C CO₂ incubator for 1 hour with rocking every 20 minutes.
- 25 5. The 1° overlay was made by preheating 25 ml M199(2X), 1.5 ml FBS, and 0.5 ml PSA at 42°C in a 50 ml Falcon tube (tube #1). Additionally,

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23 ml Agarose (0.6% in water) was heated at 42°C in a 50 ml Falcon tube (tube #2). At the end of the 1 hour incubation, tube #1 was added to tube #2 and mixed thoroughly.

6. One ml of this overlay was then added to the edge of each well.

5 7. The plate was then put in the 37°C CO₂ incubator for 4 days.

8. The 2° overlay was made by preheating 25 ml M199(2X), 1.5 ml FBS, 1.5 ml Neutral Red, and 0.5 ml PSA at 42°C in a 50 ml Falcon tube (tube #1). Additionally, 21.5 ml Agarose (0.6% in water) was heated at 42°C in a 50 ml Falcon tube (tube #2). Tube #1 was added to tube #2 and mixed
10 thoroughly.

9. One ml of this overlay was added to the center of each well.

10. It was then placed in the 37°C CO₂ incubator

Titration of P2 results

Instead of titer determination, plaques were picked for purification
15 to segregate a mixed population of large and small plaques observed. The RNA from P2 was extracted using Trizol methods according to the manufacturer's protocol, RT-PCR was performed followed by sequencing of the YF/DEN-4 prME region 5', 3' junctions, inclusive. The expected sequence of the prME was confirmed.

20

6.0 Construction of Chimeric Templates for Other Flaviviruses

Procedures for generating full-length cDNA templates encoding chimeric YF/MVE, YF/SLE, YF/WN, YF/TBE viruses are similar to those described above for the YF/DEN-2 system. Table 20 illustrates the
25 features of the strategy for generating YF 17D-based chimeric viruses. The unique restriction sites used for *in vitro* ligation, and the chimeric

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primers for engineering the C/prM and E/NSI junctions are also shown. Sources of cDNA for these heterologous flaviviruses are readily available (MVE: Dalgarno *et al.*, J. Mol. Biol. 187:309-323, 1986; SLE: Trent *et al.*, Virology 156:293-304, 1987; TBE: Mandl *et al.*, Virology 166:197-205, 5 1988; Dengue 1: Mason *et al.*, Virology 161:262-267, 1987; Dengue 2: Deubel *et al.*, Virology 155:365-377, 1986; Dengue 3: Hahn *et al.*, Virology 162:167-180, 1988; Dengue 4: Zhao *et al.*, Virology 155:77-88, 1986).

An alternative approach to engineering additional chimeric viruses 10 is to create the C/prM junction by blunt end ligation of PCR-derived restriction fragments having ends that meet at this junction and 5' and 3' termini that flank appropriate restriction sites for introduction into YF5'3'IV or an intermediate plasmid such as pBS-KS(+). The option to use a chimeric oligonucleotide or blunt-end ligation will vary, depending 15 on the availability of unique restriction sites within the envelope protein coding region of the virus in question.

6.1 Construction of YF Viruses Encoding HCV Antigens

Because the structural proteins E1 and E2 of HCV are not 20 homologous to the structural proteins of the flaviviruses described above, the strategy for expression of these proteins involves insertion within a nonessential region of the genome, such that all of these proteins are then co-expressed with yellow fever proteins during viral replication in infected cells. The region to be targeted for insertion of the proteins is the N 25 terminal portion of the NS1 protein, since the entire NS1 protein is not required for viral replication. Because of the potential problems with stability of the YF genome in the presence of heterologous sequence

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exceeding the normal size of the genome (approximately 10,000 nucleotides), the detection strategy described below can be used. In addition, deletion of NS1 may be advantageous in the chimeric YF/Flavivirus systems described above, because partial deletion of this
5 protein may abrogate the immunity to YF associated with antibodies against NS1, and thus avoid problems with vector immunity if more than one chimeric vaccine was to be needed in a given recipient, or if a YF vaccine had been previously given or needed at a future point.

The strategy involves creating a series of in-frame deletions within
10 the NS1 coding region of the YFM5.2 plasmid, in conjunction with engineering a translational termination codon at the end of E, and a series of two IRESs (internal ribosome entry sites). One IRES is immediately downstream of the termination codon and allows for expression of an open reading frame within the region between E and NS1. The second IRES
15 initiates translation from truncated NS1 proteins, providing expression of the remainder of the YF nonstructural polyprotein. These derivatives are tested for recovery of infectious virus and the construct with the largest deletion is used for insertion of foreign sequences (*e.g.*, HCV proteins) in the first IRES. This particular construct can also serve as a basis for
20 determining whether deletion of NS1 will affect vector-specific immunity in the context of YF/Flavivirus chimeric viruses expressing prM-E, as described above.

The insertion of nucleotides encoding E1, E2, and/or E1 plus E2 HCV proteins is limited by the size of the deletion tolerated in the NS1
25 protein. Because of this, truncated HCV proteins can be used to enhance stability within the modified YF clone. The HCV proteins are engineered with an N-terminal signal sequence immediately following the IRES and a

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termination codon at the C terminus. This construction will direct the HCV proteins into the endoplasmic reticulum for secretion from the cell.

The strategy for this construction is shown schematically in Fig. 21.

Plasmids encoding HCV proteins of genotype I can be used for these

5 constructions, for example, HCV plasmids obtained from Dr. Charles Rice at Washington University (Grakoui *et al.*, J. Virology 67:1385-1395, 1993), who has expressed this region of the virus in processing systems and within a replication-complement full-length HCV clone.

10 *6.2 PrM cleavage deletion mutants as attenuating vaccine candidates for flaviviruses*

Additional chimeric viruses included in the invention contain mutations that prevent prM cleavage, such as mutations in the prM cleavage site. For example, the prM cleavage site in flavivirus infectious
15 clones of interest, such as dengue, TBE, SLE, and others can be mutated by site-directed mutagenesis. Any or all of the amino acids in the cleavage site, as set forth above, can be deleted or substituted. A nucleic acid fragment containing the mutated prM-E genes can then be inserted into a yellow fever virus vector using the methods described above. The prM
20 deletion can be used with or without other attenuating mutations, for example, mutations in the E protein, to be inserted into the yellow fever virus. These mutants have advantages over single substitution mutants as vaccine candidates, because it is almost impossible to revert the deleted sequence and restore virulence.

25 The following chimeric flaviviruses of the invention were deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland, U.S.A. under the terms of the Budapest Treaty and granted a

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deposit date of January 6, 1998: Chimeric Yellow Fever 17D/Dengue Type 2 Virus (YF/DEN-2; ATCC accession number ATCC VR-2593) and Chimeric Yellow Fever 17D/Japanese Encephalitis SA14-14-2 Virus (YF/JE A1.3; ATCC accession number ATCC VR-2594).

5

Table 1Sequence comparison of JE strains and YF/JE chimeras

10	Virus	E	E	E	E	E	E	E	E	E	E
		107	138	176	177	227	243	244	264	279	315
	JE SA14-14-2	F	K	V	T	S	K	G	H	M	V
	YF/JE SA14-14-2	F	K	V	A	S	E	G	H	M	V
	YF/JE Nakayama	L	E	I	T	P	E	E	Q	K	A
15	JE Nakayama	L	E	I	T	P	E	E	Q	K	A
	JE SA14	L	E	I	T	S	E	G	Q	K	V

20

Table 2Characterization of YF/JE chimeras

	Clone	Yield (μg)	Infectivity	PBS	RNase	DNase
			plaques/100 ng LLC-MK2	log titer VERO	log titer VERO	log titer VERO
25	YF5.21v	5.5	15	7.2	0	7
	YF/JE-S	7.6	50	6.2	0	6.2
	YF/JE-N	7	60	5	0	5.4

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Table 3Plaque reduction neutralization titers on YF/JE chimeras

	Virus	non-immune ascitic fluid	YF ascitic fluid	JE ascitic fluid	non-immune IgG	YF IgG
5	YF5.2iv	<1.3	3.7	<1.3	<2.2	>4.3
	JE SA14-14-2	<1.3	<1.3	3.4	<2.2	<2.2
	YF/JE SA ₁₄ - 14-2	<1.3	<1.3	3.1	<2.2	<1.9
	YF/JE	<1.3	<1.3	3.4	<2.2	<2.2
10	Nakayama					
	JE Nakayama virus	<1.3	<1.3	3.4	not determined	not determined September 15, 1998

15

Table 4Neurovirulence of YF/JE SA14-14-2 Chimera3 week old male ICR mice

		log dose I.C.	% Mortality	
20	YF5.2iv	4	100	(7/7)
	YF/JE SA14-14-2	4	0	(0/7)
	YF/JE SA14-14-2	5	0	(0/7)
	YF/JE SA14-14-2	6	0	(0/8)

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Table 5Neuroinvasiveness of YF/JE Chimeras3 week old male ICR mice

5		log dose (intraperitoneal)	% mortality
	YF/JE Nakayama	4	0 (0/5)
	YF/JE Nakayama	5	0 (0/4)
	YF/JE Nakayama	6	0 (0/4)
	YF/JE SA14-14-2	4	0 (0/5)
10	YF/JE SA14-14-2	5	0 (0/4)
	YF/JE SA14-14-2	6	0 (0/4)

Table 615 Doses and routes of virus inoculation into groups of 4-week-old ICR mice

	Group	YF/JE s.c.	YF/JE i.c.	YF-VAX s.c.	YF-Vax i.c.	Total #
		log ₁₀ pfu	log ₁₀ pfu	log ₁₀ pfu	log ₁₀ pfu	mice
20	1	5	4.5	4.7	4.2	20
	2	4	4	4.4	3.9	20
	3	3	3	3.4	3.4	20
	4	2	2	2.4	2.4	20
	5	1	1	1.4	1.4	20
25	6	JE-Vax (BIKEN) 1:30, day 0, 7, s.c.				5
	7	JE-Vax (BIKEN) 1:300, day 0, 7, s.c.				5
	8	control s.c. (medium +10% FBS)				5
	9	control i.c. (medium +10% FBS)				5

30

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Table 7

Geometric mean neutralizing antibody titers 3 and 8 weeks after immunization,
outbred mice inoculated with graded doses of vaccines by the s.c. route

Vaccine	Dose $\log_{10}$ PFU	Antibody titer (GMT $\pm$ SD) vs.			
		JE		YF 17D	
		3w	8w	3w	8w
YF/JE	5.0	151 $\pm$ 93	5,614 $\pm$ 3514		
	4.0	38 $\pm$ 60	127 $\pm$ 247		
	3.0	19 $\pm$ 65	43 $\pm$ 560		
	2.0	7 $\pm$ 12	3 $\pm$ 71		
	1.0	2 $\pm$ 8	0		
YF 17D	4.7			2 $\pm$ 4	18 $\pm$ 13
	4.4			35 $\pm$ 24	250 $\pm$ 109
	3.4			9 $\pm$ 20	54 $\pm$ 179
	2.4			1 $\pm$ 0	53 $\pm$ 22
	1.4			0	46 $\pm$ 18

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Table 8**Immunogenicity and protection vs. challenge**

Mice were immunized on Day 0 with live vaccines and on days 0, 7, and 20 with JE-Vax, bled on day 21 and challenged on day 28.

Virus	No./group	Dose (pfu)	Route	Total no. mice
1. YF/JE (SA14-14-2 RMS)*	8	10^2 - 10^5	sc	32
2. YF 17D (iv5.2) (Vero)	8	10^2 - 10^5	sc	32
3. YF 17D (PMC)	8	10^2 - 10^5	sc	32
4. JE Nakayama	8	10^2 - 10^5	sc	32
5. JE SA14-14-2 (BHKP1)**	8	10^2 - 10^5	sc	32
6. YF/JE (Nakayama)#	8	10^2 - 10^5	sc	32
7. JE-Vax Connaught lot EJN*151B	8	100 ul 1:300 dil. on Day 0, 7 and 100 ul 1:5 dil. on D 20	sc	8
8. None (challenged)	8		ip	8
9. None (unchallenged)	8	-----	-----	8

* YF/JE SA14-14-2 vaccine candidate

** Chinese live vaccine, passed once in BHK cells

Chimeric YF/JE virus, with prM-E insert of wild-type JE Nakayama

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Table 9. Protection of C57BL/6 mice by a single SC inoculum of graded doses of live virus vaccines against IP challenge with 158 LD50 of wild-type JE virus (IC-37). Mice challenged 28 days after immunization.

Vaccine	Number of survivors/no. challenged (% survivors) by vaccine dose					
	None	1	2	3 (log10 pfu)	4	5
Yellow fever 17D (YF-Vax unpassaged)		NT*	3/8 (37.5%)	1/8 (12.5%)	1/8 (12.5%)	2/8 (25%)
Yellow fever 17D (YF-5.2iv Infect. clone)		NT	0/8 (0%)	1/8 (12.5%)	1/8 (12.5%)	1/8 (12.5%)
Yellow fever/JE SA14-14-2 chimera		1/8 (12.5%)	2/8 (25%)	7/7 (100%)	7/8 (87.5%)	7/7 (100%)
Chinese JE vaccine SA14-14-2 (BHK)		NT	1/8 (12.5%)	1/8 (12.5%)	0/8 (0%)	3/8 (37.5%)
Wild-type JE (Nakamyn)		NT	2/7 (29%)	1/6 (17%)	1/3 (33%)	1/4 (25%)
YF/JE (Nakamyn)			3/3 (100%)	5/5 (100%)	3/3 (100%)	^
Mouse brain vaccine (JE-Vax)**						7/8 (87.5%)
Control (challenge)	1/8 (12.5%)					
Control (no challenge)	8/8 (100%)					

* Not tested

^ Some mice died as a result of inoculation of the wild-type virus at high doses, thus fewer mice remain for challenge.

** Three doses at 1 week intervals

^ No mice survived initial inoculation at this dose

% Corrected from previous report

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Table 10

Geometric mean neutralizing antibody titers, C57/BL6 mice 21 days after immunization with a single SC inoculum of graded doses of live virus vaccines and 1 day after the third dose of inactivated JE-Vax.

Vaccine	Dose (log ₁₀ PFU)	Antibody titer (GMT ± SD)	
		JEV	YF 17D
YF/JE SA14-14-2	5	44.8 ± 25.2	
	4	26.5 ± 23.1	
	3	6.2 ± 4.9	
	2	1.1 ± 0.35	
	1	1 ± 0	
SA14-14-2(BHK1)	5	2.5 ± 4.3	
	4	3.5 ± 20.5	
	3	4.7 ± 15.5	
	2	1 ± 0	
JE Nakayama	5	1.32 ± 1	
	4	4 ± 4.0	
	3	1.6 ± 1.8	
	2	1 ± 0	
YF/JE-Nakayama	5	10 ± 70*	
	4	102.5 ± 45.7	
	3	76.8 ± 63.9	
	2	19.8 ± 8.1	
JE-Vax® (mouse brain)	3 doses**	2.8 ± 6.5	
YF-Vax®	5		11 ± 9.6
	4		13.8 ± 19.1
	3		4.3 ± 11.7
	2		1 ± 0
YF5.2iv (17D infect. clone)	5		29.3 ± 47.1
	4		11 ± 15.2
	3		8 ± 19.4
	2		2.1 ± 3.2
Controls	0	1 ± 0	

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* only 2/8 mice survived immunization with this virus; the low antibody titers in these animals probably reflect low level virus replication consistent with survival.

** 3 doses on days 0, 7, 20; animals were bled on day 21, 1 day after their
5 third immunization. The day 20 boost was performed with a higher dose of vaccine, thus antibody titers pre-challenge are expected to be higher than those shown here.

Table 11

10 Geometric mean neutralizing antibody titers (GMT) in 3 monkeys 2 and 4 weeks post inoculation with a single dose of YF-Vax® or ChimeriVax™ by the I.C. route

	<u>Vaccine</u>	<u>Dose (log10 pfu)</u>	<u>GMT</u>	
			<u>JE</u>	<u>YF</u>
			<u>2W</u> <u>4W</u>	<u>2W</u>
15	<u>4W</u>			
	ChimeriVax™	7.0	75 3200	
	YF-Vax®	5.0		66 4971

Table 12 Immunization and protection: rhesus monkeys

Screening HI test for flavivirus antibodies: negative

Group	N	Virus	Dose, route (log ₁₀ PFU/0.5 ml)	JE Challenge Day 60
1	3	YF/JE SA14-14-2	4.3 SC	5.0 IC
2	3	YF/JE SA14-14-2	5.3 SC	5.0 IC
3	4	Saline/sham	- SC	5.0 IC

- Viremia days 1-7 after immunization and challenge
- Neutralization test days 0, 15, 30, 45, and 60 after immunization and days 15 and 30 after challenge
- Necropsy day 30 post challenge

Table 13 Viremia, rhesus monkeys immunized with ChimeriVax™ by the SC route

Monkey	Dose log ₁₀ PFU	Day post-inoculation							
		0	1	2	3	4	5	6	
R423	4.3	<1.0*	<1.0	<1.0	1.1	1.7	1.0	<1.0	
R073		<1.0	<1.0	<1.0	1.0	1.0	<1.0	<1.0	
R364		<1.0	1.0	<1.0	1.0	1.0	<1.0	<1.0	
R756	5.3	<1.0	1.0	1.0	1.6	1.0	<1.0	<1.0	
R174		<1.0	1.3	1.8	1.6	1.1	<1.0	<1.0	
R147		<1.0	2.0	1.6	1.0	1.0	<1.0	<1.0	

* log₁₀ PFU/ml

Table 14 JE neutralizing antibody responses, rhesus monkeys immunized with ChimeriVax™ by the SC Route

50% PRNT titers, heat-inactivated serum, no added complement

Monkey	Dose log ₁₀ PFU	Day post-inoculation		
		Baseline	15	30
R423	4.3	<10	160	2560
R073		<10	80	640
R364		<10	160	320
R756	5.3	<10	20	320
R174		<10	640	2560
R147		<10	160	2560

Table 15 Protection against IC challenge, rhesus monkeys immunized with ChimeriVax™ by the SC route

Monkeys challenged IC on Day 60 with 100,000 pfu/mouse LD50

Vaccine Dose log ₁₀ PFU	No. survived/No. tested
4.3	2/3 (67%)*
5.3	3/3 (100%)
Sham	0/4 (0%)

* 1 monkey that died was a pregnant female

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Table 16

List of chimeric YF/JE mutants (1 to 9) constructed to identify residues involved in attenuation of the ChimeriVax™. Mutated amino acids on the E-proteins are shown in bold letters.

Positions	Nakayam	ChimeriVax™	Mutant Viruses										
			1	2	3	4	5	6	7	8	9	10	11
107	L	F	L	F	F	L	L	F	L	F	L	F	L
138	E	K	K	E	K	K	E	E	E	E	E	E	E
176	I	V	V	V	I	I	V	I	I	V	V	I	I
177	T	A	A	A	T	T	A	T	T	A	A	T	T
227	P	S	S	S	S	S	S	S	S	P	P	P	P
264	Q	H	H	H	H	H	H	H	H	Q	Q	Q	Q
279	K	M	M	M	M	M	M	M	M	K	K	K	K

Table 17

Dose administered i.c. (pfu)

Group	P1	P10	P18
Neat	$\geq 6 \times 10^4$	1×10^6	2×10^7
10^{-1}	$\geq 6 \times 10^3$	1×10^5	2×10^6

Table 18

Dose administered s.c. (pfu)

Group	RMS	P10	P18
Neat	2×10^5	2×10^7	3×10^7
10^5	1×10^5	5×10^5	5×10^4
10^4	1×10^4	5×10^4	5×10^3

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Table 19

Design of an experiment to determine cross-protection/interference between YF 17D and YF/JE SA14-14-2

# of female ICR mice	1st Vaccine¶	2nd vaccine		
		3 months	6 months	12 months
8	YF/JE SA14-14-2	YF-Vax®		
8	YF/JE SA14-14-2		YF-Vax®	
8	YF/JE SA14-14-2			YF-Vax®
8	JE-Vax®	YF-Vax®		
8	JE-Vax®		YF-Vax®	
8	JE-Vax®			YF-Vax®
8	YF-Vax®	YF/JE SA14-14-2		
8	YF-Vax®		YF/JE SA14-14-2	
8	YF-Vax®			YF/JE SA14-14-2
4		YF-Vax®		
4		YF/JE SA14-14-2		
4			YF-Vax®	
4			YF/JE SA14-14-2	
4				YF-Vax®
4				YF/JE SA14-14-2

¶: One dose of YF/JE SA14-14-2, $5.3 \log_{10}$ pfu/mouse, sc.

One dose of YF-Vax®, $4.4 \log_{10}$ pfu/mouse, sc.

Two doses of JE-Vax® (PMC), 0.5 ml of 1:5 dilution administered ip at 1 week intervals.

Table 20

Engineering of YF/Flavivirus chimeras

⁷ Virus	Chimeric C/prM junction ¹	Chimeric E/NS1 junction ²	5' ligation ³	3' ligation ⁴	Sites ⁵ eliminated or (created)
YF/WN	X-cactgggagagcttgaaggctc (SEQ ID NO:1)	<u>aaagccagttgcagccgcggttaa</u> (SEQ ID NO:2)	<i>AatII</i>	<i>NsiI</i>	
YF/DEN-1	X-aaggtagactggtggctccc (SEQ ID NO:3)	<u>gatcctcagttaccaaccgcggttaa</u> (SEQ ID NO:4)	<i>AatII</i>	<i>SphI</i>	<i>SphI</i> in DEN
YF/DEN-2	X-aaggtagattggtgtgcattg (SEQ ID NO:5)	<u>aaccttcagttaccaccgcggttaa</u> (SEQ ID NO:6)	<i>AatII</i>	<i>SphI</i>	
YF/DEN-3	X-aaggtgaattgaagtctcta (SEQ ID NO:7)	<u>acccccagtcaccaccgcggttaa</u> (SEQ ID NO:8)	<i>AatII</i>	<i>SphI</i>	<i>XhoI</i> in DEN (<i>SphI</i> in DEN)
YF/DEN-4	X-aaaaggaacagttgttctcta (SEQ ID NO:9)	<u>accggaagttgtcaaccgcggttaa</u> (SEQ ID NO:10)	<i>AatII</i>	<i>NsiI</i>	
YF/SLE	X-aacgtgaatagttgtagtc (SEQ ID NO:11)	<u>accgttggtcgcaccgcggttaa</u> (SEQ ID NO:12)	<i>AatII</i>	<i>SphI</i>	<i>AatII</i> in SLE
YF/MVE	X-aatttcgaagggtgaaggctc (SEQ ID NO:13)	<u>gaccggtgtttacagccgcggttaa</u> (SEQ ID NO:14)	<i>AatII</i>	<i>AgeI</i>	(<i>AgeI</i> in YF)
YF/TBE	X-tactgcgaacgacgttgccac (SEQ ID NO:15)	<u>actgggaaccttcaccgcggttaa</u> (SEQ ID NO:16)	<i>AatII</i>	<i>AgeI</i>	(<i>AgeI</i> in YF)

1,2: The column illustrates the oligonucleotide used to generate chimeric YF/Flavivirus primers corresponding to the C/prM or E/NS1 junction. (See text). X = carboxyl terminal coding sequence of the YF capsid. The underlined region corresponds to the targeted heterologous sequence immediately upstream of the *NarI* site (antisense - ccgcgg). This site allows insertion of PCR products into the Yfm5.2 (*NarI*) plasmid required for generating full-length cDNA templates. Other nucleotides are specific to the heterologous virus. Oligonucleotide primers are listed 5' to 3'.

3,4: The unique restriction sites used for creating restriction fragments that can be isolated and ligated *in vitro* to produce full-length chimeric cDNA templates are listed. Because some sequences do not contain convenient sites, engineering of appropriate sites is required in some cases (footnote 5).

5: In parentheses are the restriction enzyme sites that must be created either in the YF backbone or the heterologous virus to allow efficient *in vitro* ligation. Sites not in parentheses must be eliminated. All such modifications are done by silent mutagenesis of the cDNA for the respective clone. Blank spaces indicate that no modification of the cDNA clones is required.

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Table 22

Summary of histopathology results, monkeys inoculated with YF-Vax or YF/JE SA14-14-2 by the IC route

YF-Vax			ChimeriVax-JE		
Monkey No.	Discriminator area score	Discriminator plus target area score	Monkey No.	Discriminator area score	Discriminator plus target area score
N030	0.21	0.64	N191	0	0.17
N492	0.04	0.36	N290	0.09	0.06
N479	0	0.17	N431	0.13	0.09
Group means	0.08	0.39		0.07	0.11

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Table 23

List of initial chimeric YF/JE mutants constructed to identify residues involved in attenuation of the ChimeriVax™. Reverted amino acids on the E-proteins are shown in **BOLD**

Positions on E- Protein	Nakayam a	ChimeriVax™	MUTANT VIRUSES										
			1	2	3	4	5	6	7	8	9	10	11
107	L	F	L	F	F	L	L	F	L	F	L	F	L
138	E	K	K	E	K	K	E	E	E	E	E	E	E
176	I	V	V	V	I	I	V	I	I	V	V	I	I
177	T	A	A	A	T	T	A	T	T	A	A	T	T
227	P	S	S	S	S	S	S	S	S	P	P	P	P
264	Q	H	H	H	H	H	H	H	H	Q	Q	Q	Q
279	K	M	M	M	M	M	M	M	M	K	K	K	K

Table 24

Experiment to determine neurovirulence and neuroinvasiveness phenotypes of vaccine candidates in suckling mice

Virus	Route	AGE OF MICE (DAYS)			
		3	5	7	9
YF/Den-2	I.C.	10 ^{4*}	10 ⁴	10 ⁴	10 ⁴
YF/JESA14-14-2	I.C.	10 ⁴	10 ⁴	10 ⁴	10 ⁴
YF 17D	I.P.	10 ³	10 ³	10 ³	10 ³
MED+5%FBS	I.C., I.P.	-	-	-	-

*: PFU/0.02 ml of inoculum

Table 25 Summary of differences between virulent (Asibi) and attenuated (17D, 17DD, RMS, P18) yellow fever viruses

Gene	NT	Asibi	17D204US	RMS	P18	17D204F	17D213	17DD	AA
C	364	G	A	A	A	A	A	A	
	376	T	C	C	C	C	C	C	
	643	A	A	-	-	A	A	G	
non-M									
M	854	C	T	-	-	T	T	T	LF
	883	A	G	-	-	G	G	A	
E	1127	G	A	-	-	A	A	A	GR
	1140	C	T	-	-	T	T	C	AV
	1431	A	A	-	-	A	C	A	NT
	1436	G	G	-	-	G	G	A	DS
	1437	A	A	-	-	A	A	G	
	1452	C	T	-	-	T	T	T	AV
	1491	C	T	-	-	T	T	T	TI
	1558	C	C	-	-	C	C	A	
	1572	A	C	-	-	C	C	C	KT
	1750	C	T	-	-	T	T	T	
	1819	C	T	-	-	T	T	T	
	1870	G	A	-	-	A	A	A	MI
	1887	C	T	-	-	T	T	T	SF
	1946	C	T	-	-	T	T	C	PS
	1965	A	G	-	-	G	G	G	KR
	2110	G	G	-	-	G	G	A	
	2112	C	G	-	-	G	G	G	TR
	2142	C	A	-	-	A	A	A	PH
	2219	G	A	-	-	A	A	G	AT
	2220	C	C	-	-	C	C	T	TI
	2356	C	T	-	-	T	T	T	
NS1	2687	C	T	T	T	T	T	T	FL
	2704	A	G	G	G	G	G	G	
	3274	G	A	A	A	A	A	A	
	3371	A	G	G	G	G	G	G	VI
	3599	T	T	T	T	T	T	C	
	3613	G	A	A	A	A	A	A	
	3637	C	C	C	C	C	C	T	
ns2a	3817	G,A	G	G	G	G	G	G	
	3860	A	G	G	G	G	G	G	VM
	3915	T,A	T	T	T	T	T	T	
	4007	A	G	G	G	G	G	G	AT
	4013	C	T	T	T	T	T	C	FL
	4022	A	G	G	G	G	G	G	AT
	4025	G	G	A	A	G	G	G	VM
	4054	C	T	T	T	T	T	C	
	4056	C	T	T	T	T	T	T	FS
	4204	C	C	C	C	C	C	T	
ns2b	4289	A	C	C	C	C	C	C	LI
	4387	A	G	G	G	G	G	G	
	4505	A	C	C	C	C	C	C	LI
	4507	T	C	C	C	C	C	C	
	4507	T	C	C	C	C	C	C	
NS3	4612	T	C	C	C	C	C	T	
	4864	G,A	G	G	G	G	G	G	
	4873	T	G	G	G	G	G	T	
	4942	A	A	A	A	A	A	G	
	4957	C	C	C	C	C	C	T	
	4972	G	G	G	G	G	G	A	
	5115	A	A	A	A	A	A	A	OR
	5131	G,T	G	G	G	G	G	G	M.M.I
	5153	A	G	G	G	G	G	A	VI
	5194	T	C	C	C	C	C	C	
	5225	A	C	C	C	C	C	C	
	5363	C	C	C	C	C	C	C	
	5431	C	T	T	T	T	T	T	

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Gene	NT	Asibi	17D204US	RMS	P18	17D204F	17D213	17DD	AA
NS3	5461	T	T	C	C	T	T	T	
	5473	C	T	T	T	T	T	T	
	5641	C	A	G	G	A	G	G	
	6013	C	T	T	T	T	T	T	
	6023	C	A	T	A	A	A	A	
ns4a	6070	C	C	C	C	C	C	C	ND
	6118	C	T	T	T	T	T	T	
	6514	T	T	T	T	T	T	T	
	6529	T	C	C	C	T	T	C	
	6625	A	A	A	A	A	C	C	
	6755	A	G	G	G	A	C	C	
	6820	T	C	C	C	C	C	A	VI
	6876	T	C	C	C	C	C	C	
ns4b	7171	A	G	G	G	G	G	G	AV
	7319	G	G	A	A	A	A	A	MI
	7497	T	T	T	T	T	T	T	EK
	7571	C	A	A	A	A	C	T	LS
NS5	7580	T	C	C	C	C	C	C	
	7642	T	C	C	C	C	C	C	HY
	7701	A	G	G	G	G	G	G	
	7945	C	T	T	T	T	T	T	RO
	7975	C	C	C	C	C	C	T	
	8008	T	C	C	C	C	C	T	
	8029	T	T	C	C	C	C	C	
	8212	C	C	T	T	T	T	C	
	8581	A	A	C	C	A	A	C	
	8629	C	T	T	T	T	T	T	
	8808	A	A	A	A	A	A	T	
	9397	A	A	A	A	A	A	G	NS
	9605	A	G	G	G	A	A	G	
	10075	G,T	G	G	G	A	A	A	DN
	10142	G	A	A	A	A	G	G	MMJ
	10243	G	A	A	A	A	A	A	KE
	10285	T	C	C	C	C	C	A	
	10312	A	G	G	G	G	G	C	
	10316	T,C	T	T	T	T	T	G	
3' NC	10339	C	G	G	G	G	G	G	SSP
	10367	T	C	C	C	C	C	C	
	10418	T	C	C	C	C	C	C	
	10454	A	G	A	A	A	A	C	
	10550	T	C	C	C	C	C	A	
	10722	G	G	G	G	A	G	T	
	10800	G	A	A	A	A	A	G	
	10847	A	C	C	C	C	C	A	

NT: nucleotide numbers are from 5' terminus of the genome. Where clonal differences were present, both nucleotides as well as amino acids (if appropriate) are shown. If nucleotide change results in an amino acid substitution, the amino acid (AA) is shown from left to right (e.g. from Asibi to 17D). -: The genes for prME in RMS (YF17D/JESA14-14-2) and P18 (passage 18th of the RMS) are from JEV strain SA14-14-2, therefore not comparable with YFV sequences. Sequences for Asibi is taken from Hahn et al. 1987. 17D204US from Rice et al. 1985, 17D204F from Dupuy et al. 1989. RMS and P18 are unpublished sequences (Oravax, Inc.), 17D213 and 17DD from Duarte dos Santos et al. 1994. Note that there is no sequence difference between RMS and passage 18th. There are 5 nucleotide differences (nucleotide positions are shaded) between published YF17D sequence and RMS shown in bold letters; Changes in 5461, 5641, 8212 and 8581 are silent and do not result in amino acid substitution. Changes in positions 4025 and 7319 result in amino acid substitution.

Table 26. Immunogenicity of ChimeriVax™-D2 passed in Vero cells for mice

Passage level ^a	Dose (Log ₁₀ pfu)	GMT ^b	
		SC	IC
P3	5	1 ± 0 ^c	61 ± 47
	4	1 ± 0	7 ± 15
P5	5	1 ± 0	46 ± 16
	4	1 ± 0	9 ± 20
P10	5	1.8 ± 7.7	46 ± 53
	4	1 ± 0	7 ± 15
P18	5	1 ± 0	53 ± 17
	4	1 ± 0	2 ± 16

a: ChimeriVax™-Den2 virus was passaged in Vero _{PM} cells (P141-147) at MOI of 0.1-0.5 and harvested 2-3 days PI.

B: Geometric Mean Titers measured as the last dilution of sera which resulted in 50% reduction in number of virus plaques.

C: Titers less than 1:10

Table 27. Immunization and challenge of yellow fever immune monkeys

1 st Vaccine	2 nd Vaccine	Seroconversion		Viremic after wt Den2 challenge
		Den2	YF	
YF 17D	ChimeriVax-Den2	3/3	3/3	0/3
YF 17D	Dengue-2 wt	4/4	4/4	0/4
YF 17D	YF 17D	0/3	3/3	3/3
YF 17D	None	0/2	2/2	2/2
None	None	0/2	0/2	2/2

Table 28. Viremia in rhesus monkeys inoculated SC with graded doses of ChimeriVax™-Den-2 virus.

Virus	Dose (Log ₁₀ PFU)	No. viremic (%)	Duration (days) mean	Titer mean peak
ChimeriVax™-Den-2	2.0	4/4 (100)	3.2	1.6
	3.0	5/5 (100)	3.8	1.6
	4.0	5/5 (100)	4.0	1.3
	5.0	4/4 (100)	4.3	1.4
Dengue-2 (18603)	4.0	4/4(100)	5	3.6

Table 29. Neutralizing antibody responses in monkeys inoculated with graded doses of ChimeriVax™-Den-2 and protection against wild-type dengue-2 challenge

Dose Log ₁₀ PFU	No. seroconverted	GMT by day			No. Viremic after Challenge (D60)
		0	15	30	
2.0	4/4	<10	47	316	0/4
3.0	5/5	<10	112	275	0/5
4.0	5/5	<10	269	316	0/5
5.0	4/4	<10	631	376	0/4
Sham	0/4	<10	<10	<10	4/4

Table 30. Mean virus titer in orally exposed mosquitoes 22 days post feeding

Mosquito Species	Virus	# Infected/ # Fed	Mean Titer*
<i>Ae. aegypti</i>	JE SA14-14-2	20/20	6.0
	YF 17D	0/30	< 1.0
	ChimeriVax™-JE	0/40	< 1.0
<i>Ae. albopictus</i>	JE SA14-14-2	38/38	5.8
	YF 17D	0/44	<1.0
	ChimeriVax™-JE	0/56	<1.0

*log₁₀pfu/ml

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Table 31

Primers (restriction sites are underlined)

#1) YFM5'3'(4.56)+

(GTGAGCATTGAGAAAGCGCCACGCTTC)(SEQ ID NO:17)

#2) YF0.481-

(TCCACCCGTCATCAACAGCATTCCCAAAATTAG)(SEQ ID NO:18)

#3) 1DE 0.42+

(GAATGCTGTTGATGACGGGTGGATTTTCATCTGACCACACGAGGG)
(SEQ ID NO:19)

#4) 1DE 1.095-

(NheI/BstBI)(GCCGCTAGCTTTTCGAAGGACGGCAGGGTTTGTGACT
TC)(SEQ ID NO:20)

#5) 1DE 1.102+

(BstBI)(GCCATGCATTTTCGAAAACGTGCATCGAAGCTAAAATAT
C)(SEQ ID NO:21)

#7) 1DE 2.409FUSE-

(GGCGCATCCTTGATCGGCGCCAACCATGACTCCTAGGTACAG)(SE
Q ID NO:22)

#8) YF NarI+

(GGCGCCGATCAAGGATGCGCCATC)(SEQ ID NO:23)

#9) YF 8.545-

(CCAAGAGGTCATGTACTCAG)(SEQ ID NO:24)

#10) SP6YFa+

(ATTTAGGTGACACTATAGAGTAAATCCTGTGTGCTAATT)(SEQ ID
NO:25)

Table 32

Oligonucleotide primers.

No.	Name	Sequence
1	3D 0.432+	5'-GAATGCTGTTGATGACGGGTGGATTCCACTTAACTTCACGAG ATGG (SEQ ID NO:26)
2	3DE 1.095-	5'-GCCGCTAGCCTTTCGAAGGGTCGCCAGCTGAGTGGCCTC (SEQ ID NO:27)
3	3DE 1.102+	5'-GCCGCTAGCTTCGAAAGCTATGCATTGAGGGAAAAATTAC (SEQ ID NO:28)
4	3DE 2.409-	5'-GCCGCCGGCGCCACCACGACCCCCAGATAGAGTG (SEQ ID NO:29)
5	YFM5'3'(4.65)+	5'-GTGAGCATTGAGAAAGCGCCACGCTTC (SEQ ID NO:30)
6	YF 0.481-	5'-TCCACCCGTCATCAACAGCATTCCCAAATTAG (SEQ ID NO:31)
7	YF 0.2+	5'-ATGGTACGACGAGGAGTTCGC (SEQ ID NO:32)
8	Kpd3/-Xho/+	5'-GGTTGATGTGGTGCTGGAGCACGGTGGGTGTG (SEQ ID NO:33)
9	PsD3/ .7+	5'-TACATCGACATGGGTGAC (SEQ ID NO:34)
10	KPsD3/ .75-	5'-GACATGGGGAGCTAACGC (SEQ ID NO:35)
11	KPsD3/ 1.5-	5'-CCCGAGGGTTCCATATTCAGG (SEQ ID NO:36)
12	KPsD3/ 1.6+	5'-GGAACAGGAAAGAGCTTC (SEQ ID NO:37)
13	KPsD3/ 1.9-	

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- 5'-GAGTATTGTCCCATGCTG (SEQ ID NO:38)
- 14 KPsD3/ 2.1+
5'-GGAATTGGAGACAAAGCC (SEQ ID NO:39)
- 15 KPs5.2 0.23+
5'-TGGATAGTGGACAGACAGTGG (SEQ ID NO:40)
- 16 KPs5.2 1.66-
5'-CTCTAAATATGAAGATACCATC (SEQ ID NO:41)
- 17 SP6-yfa
5'-ATTTAGGTGACACTATAGAGTAAATCCTGTGTGCTAATT
(SEQ ID
NO:42)

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Table 33

Amino acid differences in the prM-E region of DEN3 H87 and PaH881/88 strains

Position (nt/gene) ¹	H87(PaH881/88 (codon change)	Position (a.a.) ²	H87(PaH881/88 (a.a. change)
599/ prM	CAC→CTC	168	H→L
605/ prM	ACC→GCC	171	T→A
932/ prM	ACA→GCA	280	T→A
1373/ E	GAC→AAC	427	D→N
1394/ E	GAA→GAC	434	E→D
1424/ E	TCC→CCC	444	S→P
1439/ E	GCT→GTT	449	A→V
1607/ E	AAA→GAG	505	K→E
1742/ E	ACC→AAC	550	T→N
1805/ E	AAA→GAA	571	K→E
2105/ E	AGG→AAG	671	R→K
2366/ E	ATT→GTT	758	I→V

1 Positions of nucleotides in the DEN3 genome; numbering is according to (Osatomi *et al.*, Virology 176:643-647, 1990)

2 Positions of amino acid residues in the DEN3 polyprotein; numbering is according to (Osatomi *et al.*, Virology 176:643-647, 1990).

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Table 34

- #1) YFM5'3'(4.56)+ (GTGAGCATTGAGAAAGCGCCACGCTTC)(SEQ ID NO:43)
- #2) YF0.481- (TCCACCCGTCATCAACAGCATTCCCAAATTAG)(SEQ ID NO:44)
- #3) 4DE 0.432+
(GAATGCTGTTGATGACGGGTGAATTTACCTGTCAACAAGAGACGG)
(SEQ ID NO:45)
- #4) 4DEE 1.095-
(GCCGCTAGCGGTTTCGAAATAGAGCCACTTCCTTGGCTGT)(SEQ ID NO:46)
- #5) 4DE 1.102+
(GCCGCTAGCTTCGAACCTATTGCATTGAAGCCTCGATATC)(SEQ ID NO:47)
- #6) 4DE 2.409-
(GCCGCCGGCGCCAACTGTGAAACCTAGAAACACAG)(SEQ ID NO:48)
- #7)
sp6YFa+(ATTTAGGTGACACTATAGAGTAAATCCTGTGTGCTAATT)(SEQ ID NO:49)

Other Embodiments

Other embodiments are within the following claims. For example, the prM-E protein genes of other flaviviruses of medical importance can be inserted into the yellow fever vaccine virus backbone to produce

5 vaccines against other medically important flaviviruses (see, *e.g.*, Monath *et al.*, "Flaviviruses," In *Virology*, Fields (ed.), Raven-Lippincott, New York, 1995, Volume I, 961-1034). Examples of additional flaviviruses from which genes to be inserted into the chimeric vectors of the invention can be obtained include, *e.g.*, Kunjin, Central European Encephalitis,

10 Russian Spring-Summer Encephalitis, Powassan, Kyasanur Forest Disease, and Omsk Hemorrhagic Fever viruses. In addition, genes from even more distantly related viruses can be inserted into the yellow fever vaccine virus to construct novel vaccines.

Vaccine Production and Use

15 The vaccines of the invention are administered in amounts, and by using methods, that can readily be determined by persons of ordinary skill in this art. The vaccines can be administered and formulated, for example, in the same manner as the yellow fever 17D vaccine, *e.g.*, as a clarified suspension of infected chicken embryo tissue, or a fluid harvested from

20 cell cultures infected with the chimeric yellow fever virus. Thus, the live, attenuated chimeric virus is formulated as a sterile aqueous solution containing between 100 and 1,000,000 infectious units (*e.g.*, plaque-forming units or tissue culture infectious doses) in a dose volume of 0.1 to 1.0 ml, to be administered by, for example, intramuscular, subcutaneous,

25 or intradermal routes. In addition, because flaviviruses may be capable of infecting the human host *via* the mucosal routes, such as the oral route

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(Gresikova *et al.*, "Tick-borne Encephalitis," In *The Arboviruses, Ecology and Epidemiology*, Monath (ed.), CRC Press, Boca Raton, Florida, 1988, Volume IV, 177-203), the vaccine virus can be administered by a mucosal route to achieve a protective immune response. The vaccine can be

5 administered as a primary prophylactic agent in adults or children at risk of flavivirus infection. The vaccines can also be used as secondary agents for treating flavivirus-infected patients by stimulating an immune response against the flavivirus.

It may be desirable to use the yellow fever vaccine vector system

10 for immunizing a host against one virus (for example, Japanese Encephalitis virus) and to later reimmunize the same individual against a second or third virus using a different chimeric construct. A significant advantage of the chimeric yellow fever system is that the vector will not elicit strong immunity to itself. Nor will prior immunity to yellow fever

15 virus preclude the use of the chimeric vaccine as a vector for heterologous gene expression. These advantages are due to the removal of the portion of the yellow fever vaccine E gene that encodes neutralizing (protective) antigens to yellow fever, and replacement with another, heterologous gene that does not provide cross-protection against yellow fever. Although YF

20 17D virus nonstructural proteins may play a role in protection, for example, by eliciting antibodies against NS1, which is involved in complement-dependent antibody mediated lysis of infected cells (Schlesinger *et al.*, J. Immunology 135:2805-2809, 1985), or by inducing cytotoxic T cell responses to NS3 or other proteins of the virus, it is

25 unlikely that these responses will abrogate the ability of a live virus vaccine to stimulate neutralizing antibodies. This is supported by the facts that (1) individuals who have been previously infected with JE virus respond to vaccination with YF 17D similarly to persons without previous

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JE infection, and (2) individuals who have previously received the YF 17D vaccine respond to revaccination with a rise in neutralizing antibody titers (Sweet *et al.*, Am. J. Trop. Med. Hyg. 11:562-569, 1962). Thus, the chimeric vector can be used in populations that are immune to yellow fever because of prior natural infection or vaccination, and can be used repeatedly, or to immunize simultaneously or sequentially with several different constructs, including yellow fever chimeras with inserts from, for example, Japanese Encephalitis, St. Louis Encephalitis, or West Nile viruses.

10 For vaccine applications, adjuvants that are known to those skilled in the art can be used. Adjuvants that can be used to enhance the immunogenicity of the chimeric vaccines include, for example, liposomal formulations, synthetic adjuvants, such as saponins (*e.g.*, QS21), muramyl dipeptide, monophosphoryl lipid A, or polyphosphazine. Although these
15 adjuvants are typically used to enhance immune responses to inactivated vaccines, they can also be used with live vaccines. In the case of a chimeric vaccine delivered via a mucosal route, for example, orally, mucosal adjuvants such as the heat-labile toxin of *E. coli* (LT) or mutant derivations of LT are useful adjuvants. In addition, genes encoding
20 cytokines that have adjuvant activities can be inserted into the yellow fever vectors. Thus, genes encoding cytokines, such as γ -interferon, GM-CSF, IL-2, IL-12, IL-13, or IL-5, can be inserted together with heterologous flavivirus genes to produce a vaccine that results in enhanced immune responses, or to modulate immunity directed more specifically
25 towards cellular, humoral, or mucosal responses.

In addition to vaccine applications, as one skilled in the art can readily understand, the vectors of the invention can be used in gene

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therapy methods to introduce therapeutic gene products into a patient's cells and in cancer therapy. In these methods, genes encoding therapeutic gene products are inserted into the vectors, for example, in place of the gene encoding the prM-E protein.

- 5 Yellow fever 17D virus targets cells of the lymphoid and reticuloendothelial systems, including precursors in bone marrow, monocytes, macrophages, T cells, and B cells (Monath, "Pathobiology of the Flaviviruses," pp. 375-425, in Schlesinger & Schlesinger (Eds.), *The Togaviridae and Flaviviridae*, Plenum Press, New York 1986). The
- 10 yellow fever 17D virus thus naturally targets cells involved in antigen presentation and immune stimulation. Replication of the virus in these cells, with high-level expression of heterologous genes, makes yellow fever 17D vaccine virus an ideal vector for gene therapy or
- 15 immunotherapy against cancers of the lymphoreticular system and leukemias, for example. Additional advantages are that (1) the flavivirus genome does not integrate into host cell DNA, (2) yellow fever virus appears to persist in the host for prolonged periods, and (3) that heterologous genes can be inserted at the 3' end of the yellow fever vector, as described above in the strategy for producing a Hepatitis C vaccine.
- 20 Yellow fever 17D virus can be used as a vector carrying tumor antigens for induction of immune responses for cancer immunotherapy. As a second application, yellow fever 17D can be used to target lymphoreticular tumors and express heterologous genes that have anti-tumor effects, including cytokines, such as TNF-alpha. As a third
- 25 application, yellow fever 17D can be used to target heterologous genes to bone marrow to direct expression of bioactive molecules required to treat hematologic diseases, such as, for example, neutropenia; an example of a bioactive molecule that can be used in such an application is GM-CSF, but

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other appropriate bioactive molecules can be selected by those skilled in the art.

An additional advantage of the yellow fever vector system is that flaviviruses replicate in the cytoplasm of cells, so that the virus replication strategy does not involve integration of the viral genome into the host cell (Chambers *et al.*, "Flavivirus Genome Organization, Expression, and Replication," In *Annual Review of Microbiology* 44:649-688, 1990), providing an important safety measure.

All references cited herein are incorporated by reference in their entirety.

What is claimed is:

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1. A chimeric live, infectious, attenuated virus, comprising:

a yellow fever virus in which the nucleotide sequence encoding a prM-E protein is either deleted, truncated, or mutated so that functional yellow fever virus prM-E protein is not expressed, and

5 integrated into the genome of said yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that said prM-E protein of said second flavivirus is expressed.

2. The chimeric virus of claim 1, wherein said second flavivirus is selected from the group consisting of a Japanese Encephalitis (JE) virus, a
10 Dengue virus selected from the group consisting of Dengue types 1, 2, 3, and 4, a Murray Valley Encephalitis virus, a St. Louis Encephalitis virus, a West Nile virus, a Tick-borne Encephalitis virus (*i.e.*, a Central European Encephalitis virus or a Russian Spring-Summer Encephalitis virus), a Hepatitis C virus, a Kunjin virus, a Powassan virus, a Kyasanur Forest
15 Disease virus, and an Omsk Hemorrhagic Fever virus.

3. The chimeric virus of claim 1, wherein said second flavivirus is a Dengue virus, and nucleotide sequences derived from said Dengue virus are derived from two or more different Dengue strains.

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4. The chimeric virus of claim 1, wherein the nucleotide sequence encoding the prM-E protein of said second, different flavivirus replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus or comprises a mutation that prevents prM cleavage to produce M
5 protein.

5. The chimeric virus of claim 1, wherein the prM signal of said chimeric virus is that of yellow fever virus.

6. The chimeric virus of claim 1, wherein the NS2B-NS3 protease recognition site and the signal sequences and cleavage sites at the C/prM
10 and E/NS1 junctions are maintained in construction of said chimeric flavivirus.

7. Use of a chimeric, live, infectious attenuated virus in the preparation of a medicament for preventing or treating flavivirus infection in a patient, said virus comprising:

15 a yellow fever virus in which the nucleotide sequence encoding a prM-E protein is either deleted, truncated, or mutated so that functional yellow fever virus prM-E protein is not expressed, and

integrated into the genome of said yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so
20 that said prM-E protein of said second flavivirus is expressed.

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8. The use of claim 7, wherein said second flavivirus is selected from the group consisting of a Japanese Encephalitis (JE) virus, a Dengue virus selected from the group consisting of Dengue types 1, 2, 3, and 4, a Murray Valley Encephalitis virus, a St. Louis Encephalitis virus, a West Nile virus, a Tick-borne Encephalitis virus (*i.e.*, a Central European Encephalitis virus or a Russian Spring-Summer Encephalitis virus), a Hepatitis C virus, a Kunjin virus, a Powassan virus, a Kyasanur Forest Disease virus, and an Omsk Hemorrhagic Fever virus.

9. The use of claim 7, wherein second flavivirus is a Dengue virus, and nucleotide sequences derived from said Dengue virus are derived from two or more different Dengue strains.

10. The use of claim 7, wherein the nucleotide sequence encoding the prM-E protein of said second, different flavivirus replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus or comprises a mutation that prevents prM cleavage to produce M protein.

11. The use of claim 7, wherein the prM signal of said chimeric virus is that of yellow fever virus.

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12. The use of claim 7, wherein the NS2B-NS3 protease recognition site and the signal sequences and cleavage sites at the C/prM and E/NS1 junctions are maintained in construction of said chimeric flavivirus.

5 13. A nucleic acid molecule encoding a chimeric live, infectious, attenuated virus comprising:

 a yellow fever virus in which the nucleotide sequence encoding a prM-E protein is either deleted, truncated, or mutated so that functional yellow fever virus prM-E protein is not expressed, and

10 integrated into the genome of said yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that said prM-E protein of said second flavivirus is expressed.

 14. The nucleic acid molecule of claim 13, wherein said second flavivirus is selected from the group consisting of a Japanese Encephalitis
15 (JE) virus, a Dengue virus selected from the group consisting of Dengue types 1, 2, 3, and 4, a Murray Valley Encephalitis virus, a St. Louis Encephalitis virus, a West Nile virus, a Tick-borne Encephalitis virus (*i.e.*, a Central European Encephalitis virus or a Russian Spring-Summer Encephalitis virus), a Hepatitis C virus, a Kunjin virus, a Powassan virus, a
20 Kyasanur Forest Disease virus, and an Omsk Hemorrhagic Fever virus.

-151-

15. The nucleic acid molecule of claim 13, wherein second flavivirus is a Dengue virus, and nucleotide sequences derived from said Dengue virus are derived from two or more different Dengue strains.

16. The nucleic acid molecule of claim 13, wherein the nucleotide
5 sequence encoding the prM-E protein of said second, different flavivirus replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus or comprises a mutation that prevents prM cleavage to produce M protein.

17. The nucleic acid molecule of claim 13, wherein the prM signal
10 of said chimeric virus is that of yellow fever virus.

18. The nucleic acid molecule of claim 13, wherein NS2B-NS3 protease recognition site and the signal sequences and cleavage sites at the C/prM and E/NS1 junctions are maintained in construction of said chimeric flavivirus.

15 19. Use of a yellow fever virus vector comprising a gene encoding a gene product in the preparation of a medicament for producing said gene product in a cell of a patient.

20. The use of claim 19, wherein said cell is a cell of the lymphoid system or the reticuloendothelial system, or a precursor thereof.

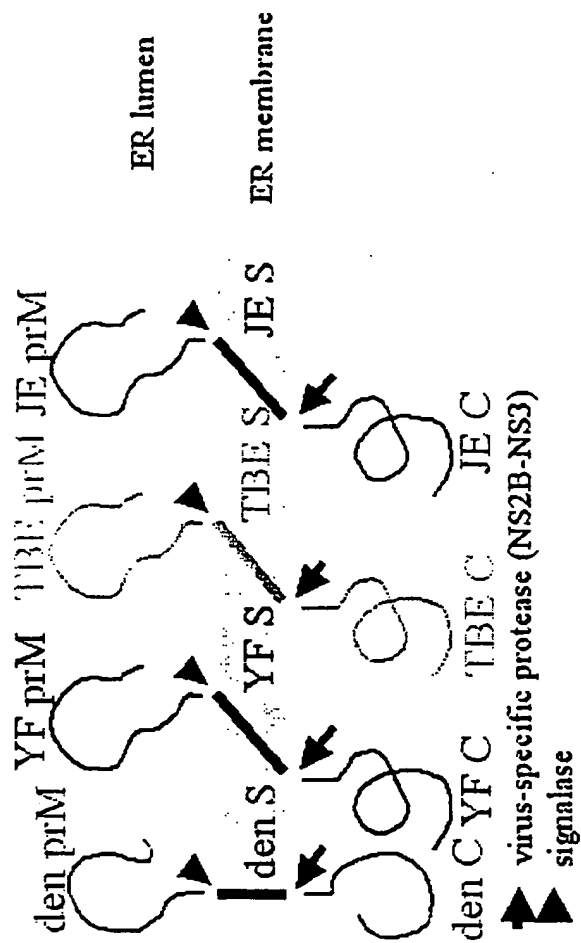
-152-

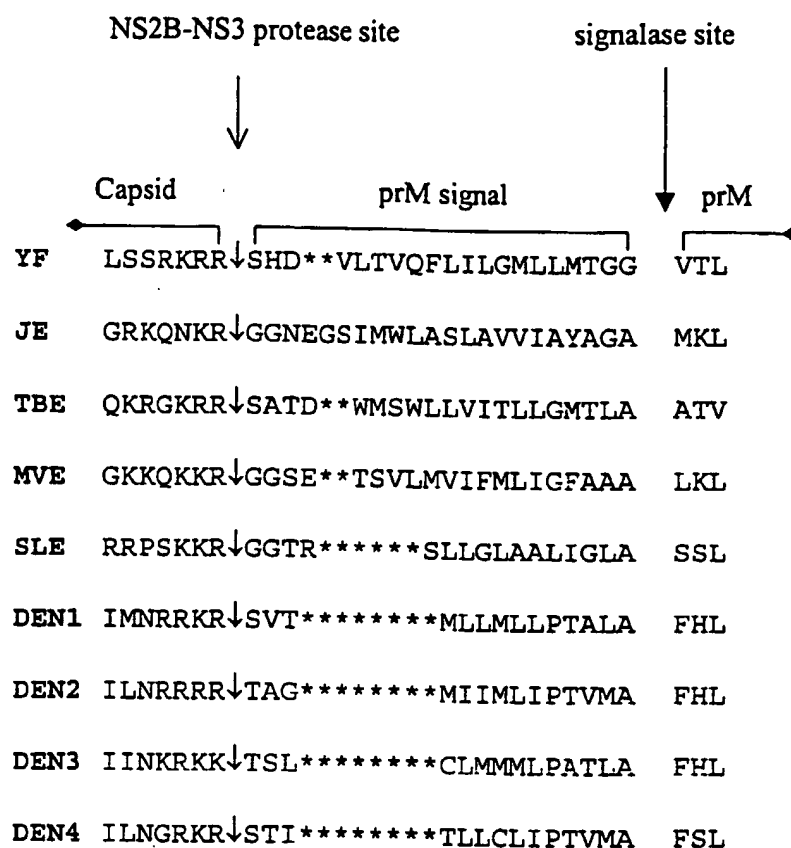
21. The use of claim 19, wherein said patient has cancer, such as leukemia.

22. The use of claim 19, wherein said gene product is a tumor antigen or a cytokine.

Fig. 1A

Processing events at the C/prM junction of the parental viruses





Virus names abbreviations

YF = yellow fever

JE = Japanese encephalitis

TBE = Tick-borne encephalitis

MVE = Murray Valley encephalitis

SLE = Saint Louis encephalitis

DEN1-4 = dengue serotypes 1-4

Fig. 1B

Fig. 2

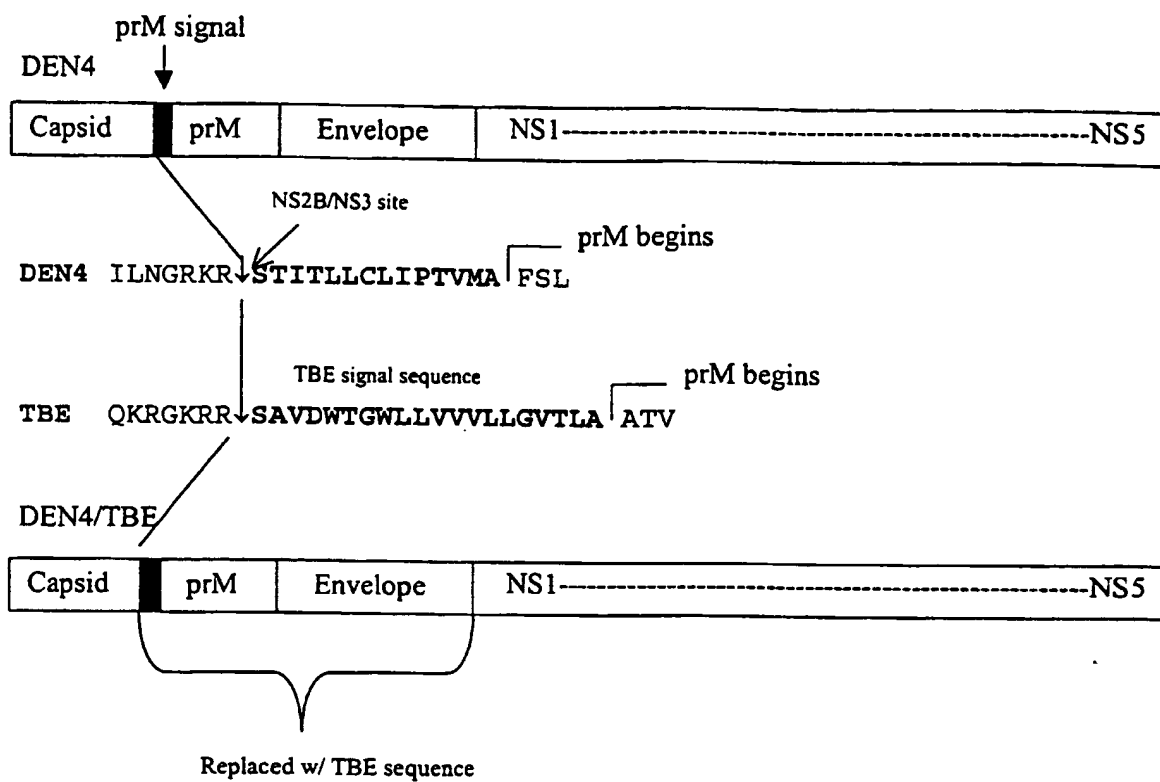


Fig. 3

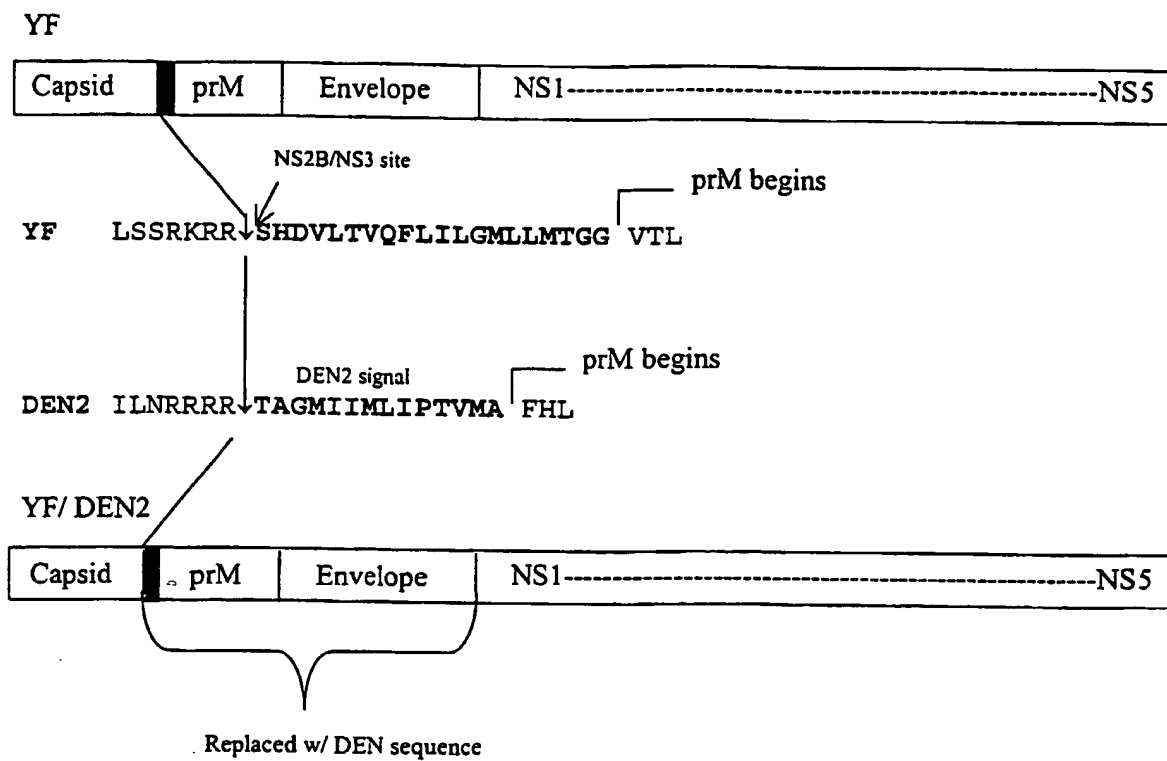


Fig. 4

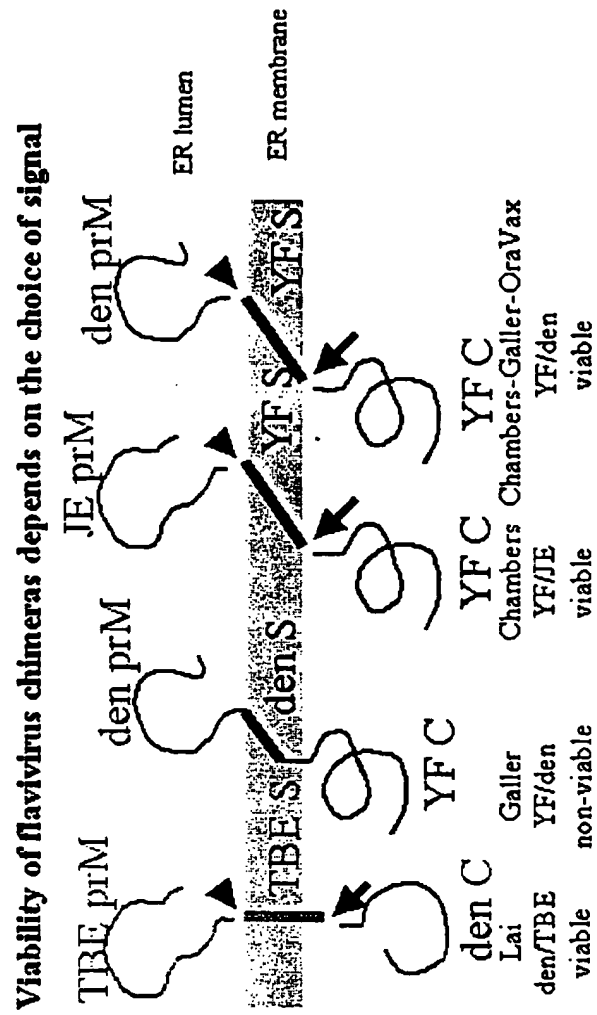
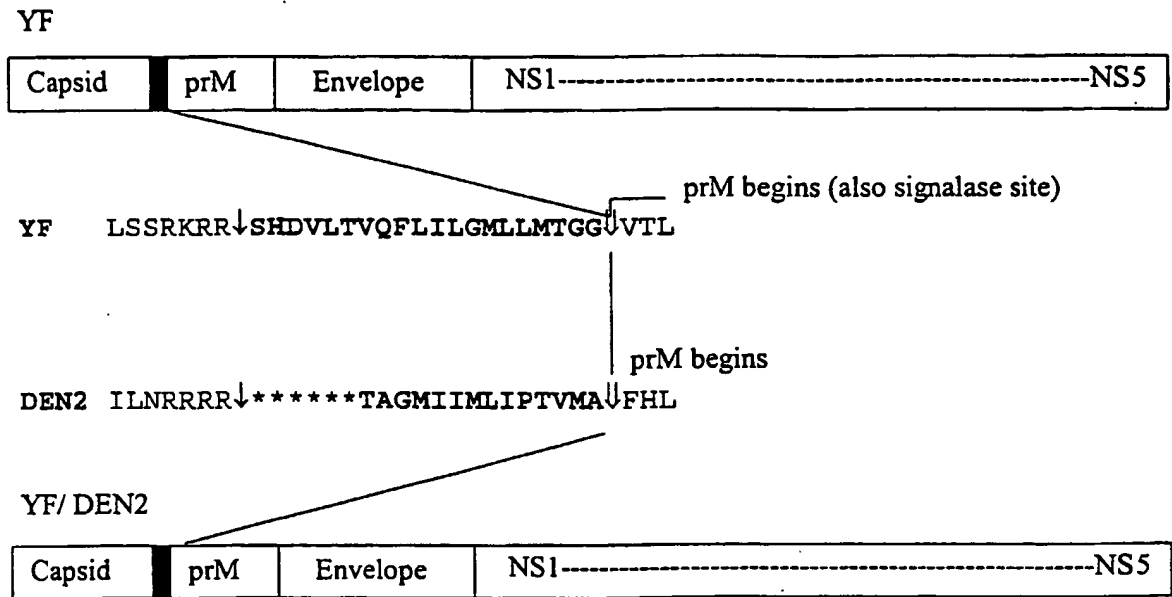


Fig. 5



Junction sequences of ChimeriVax™-JE (YF/JE) virus

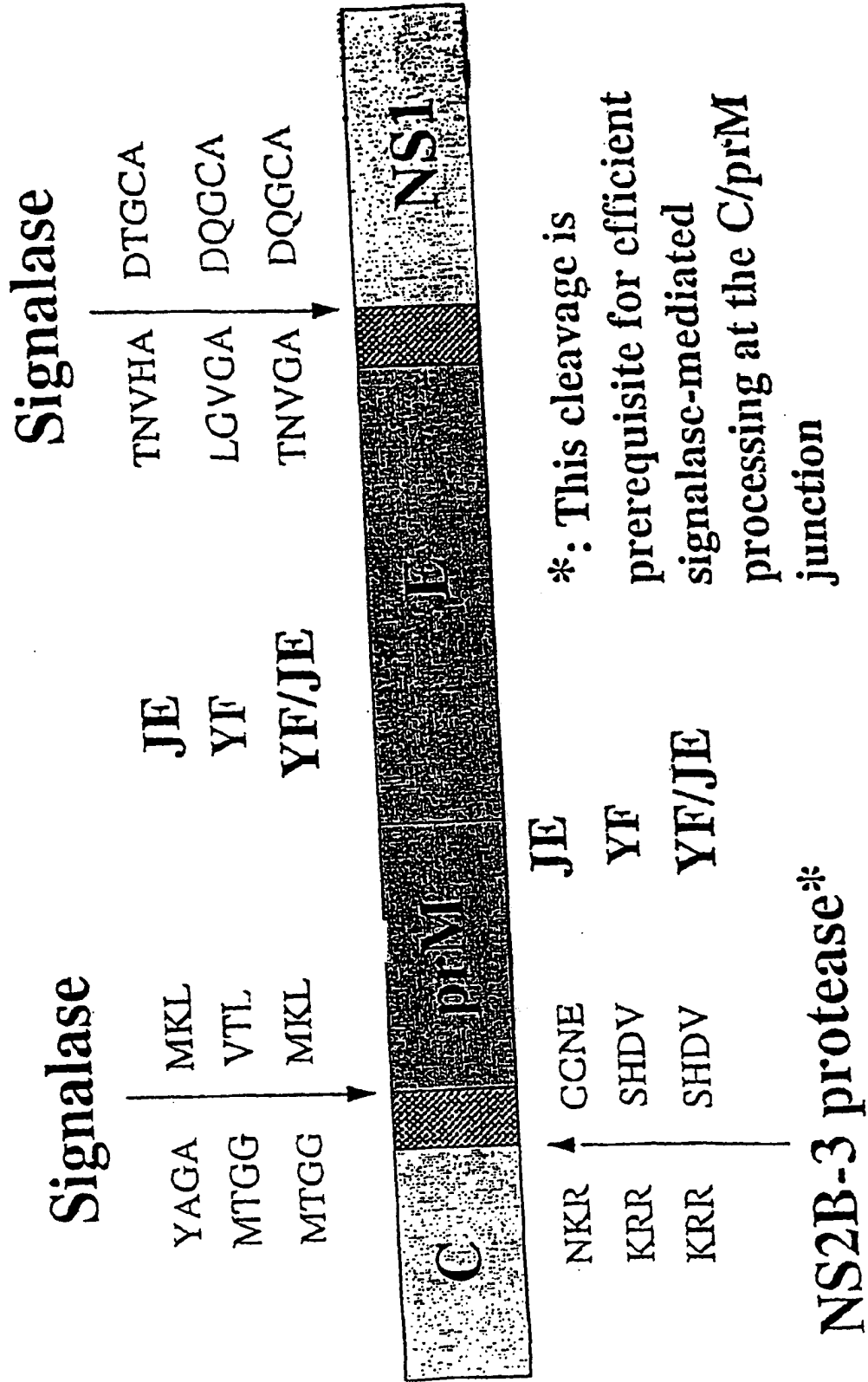


Fig. 6

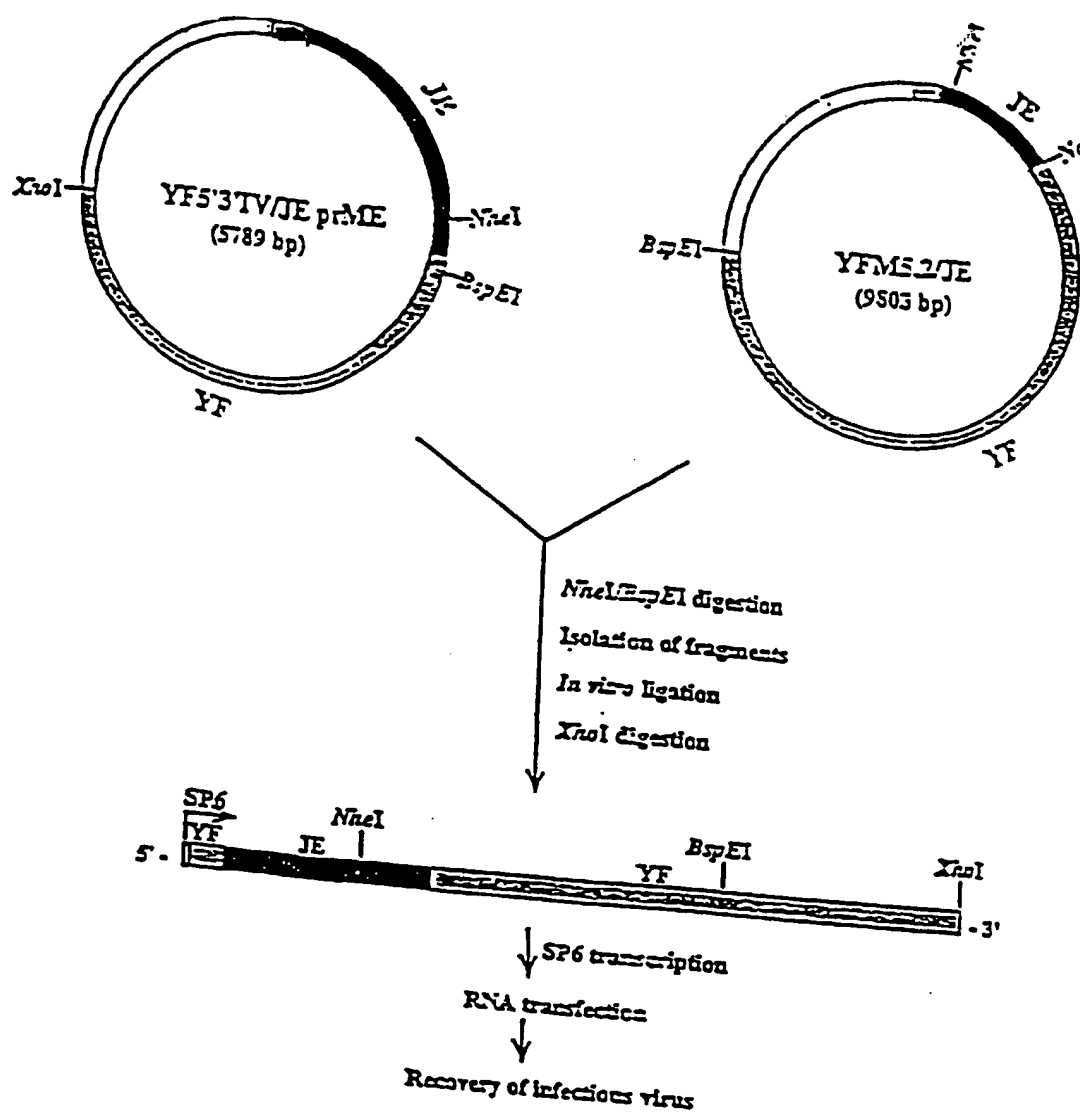


Fig. 7

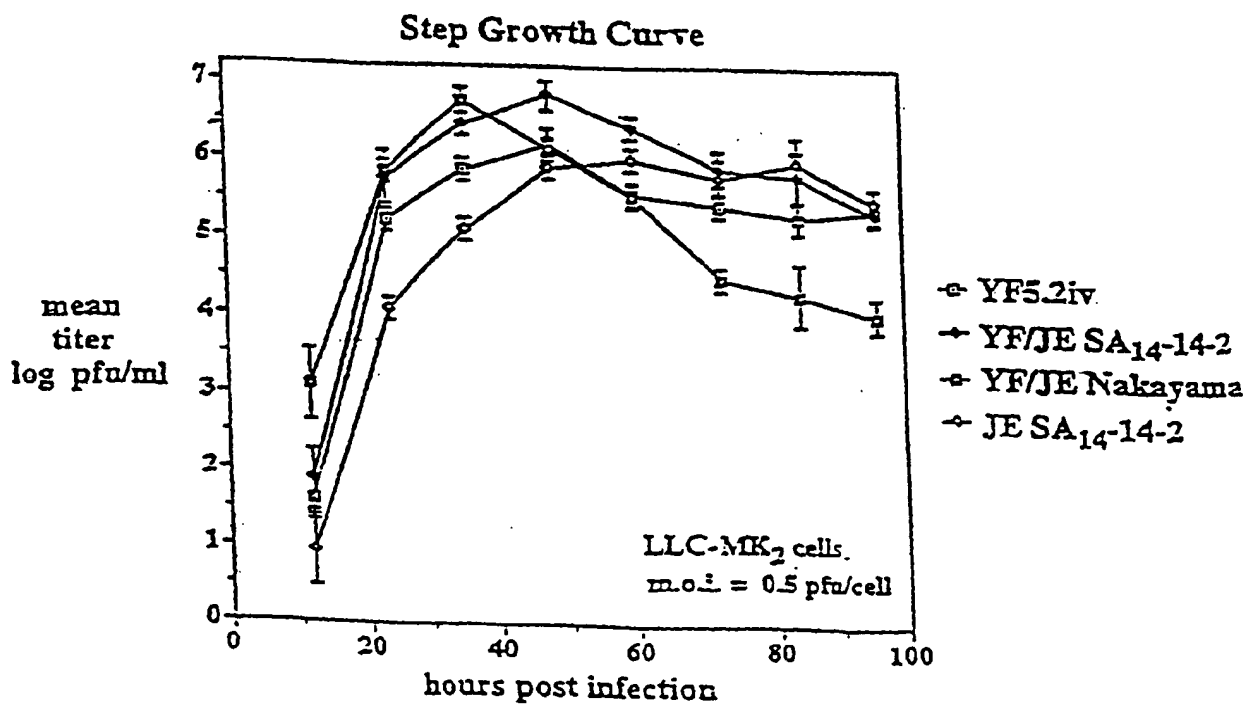
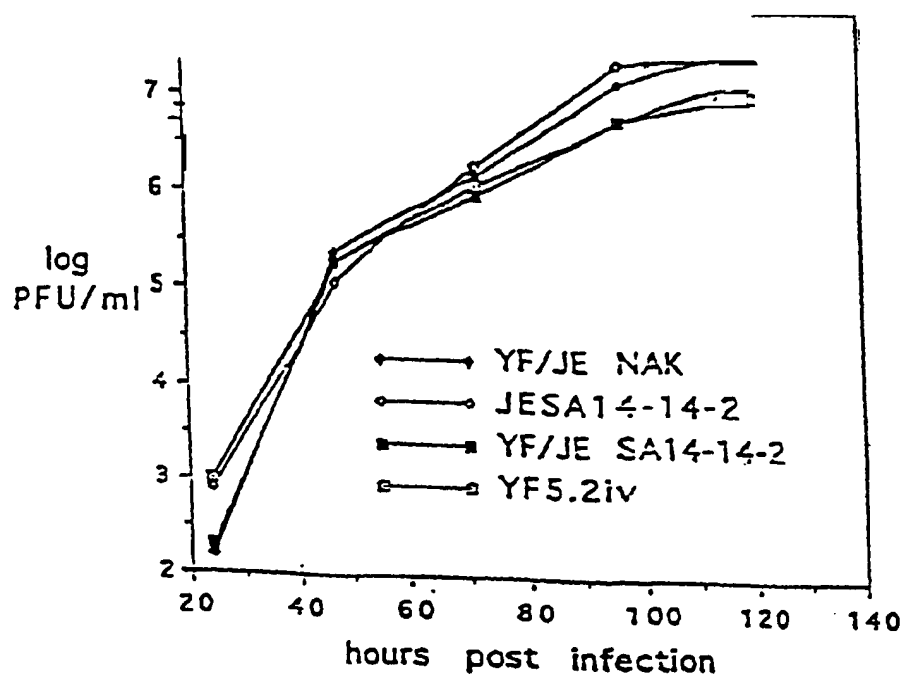
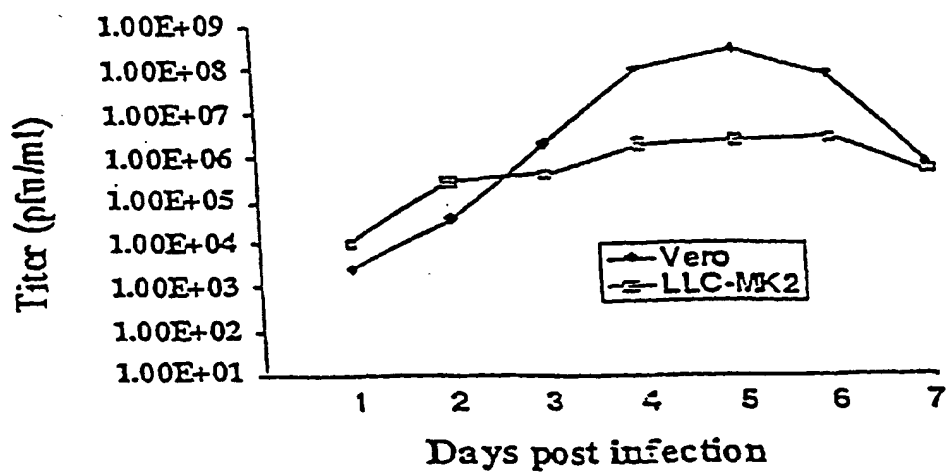
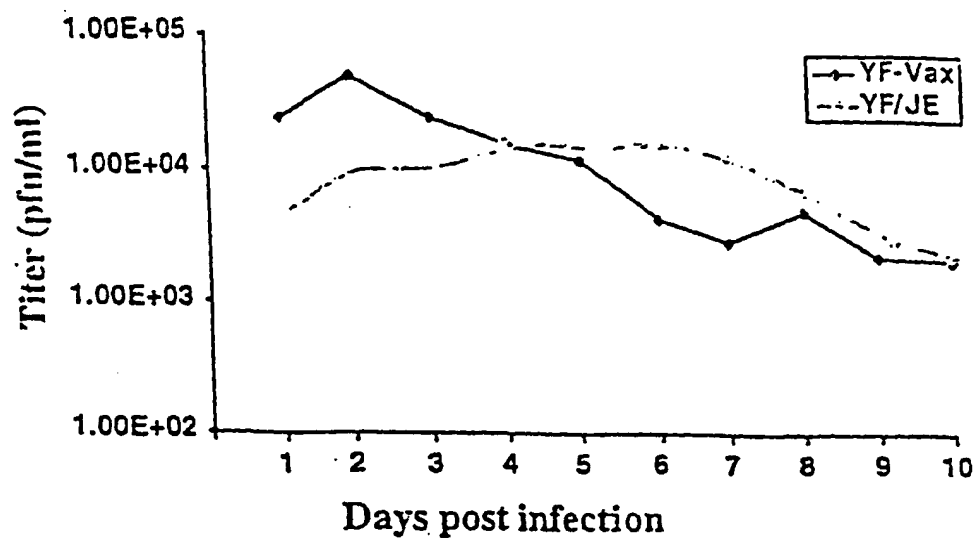


Fig. 8



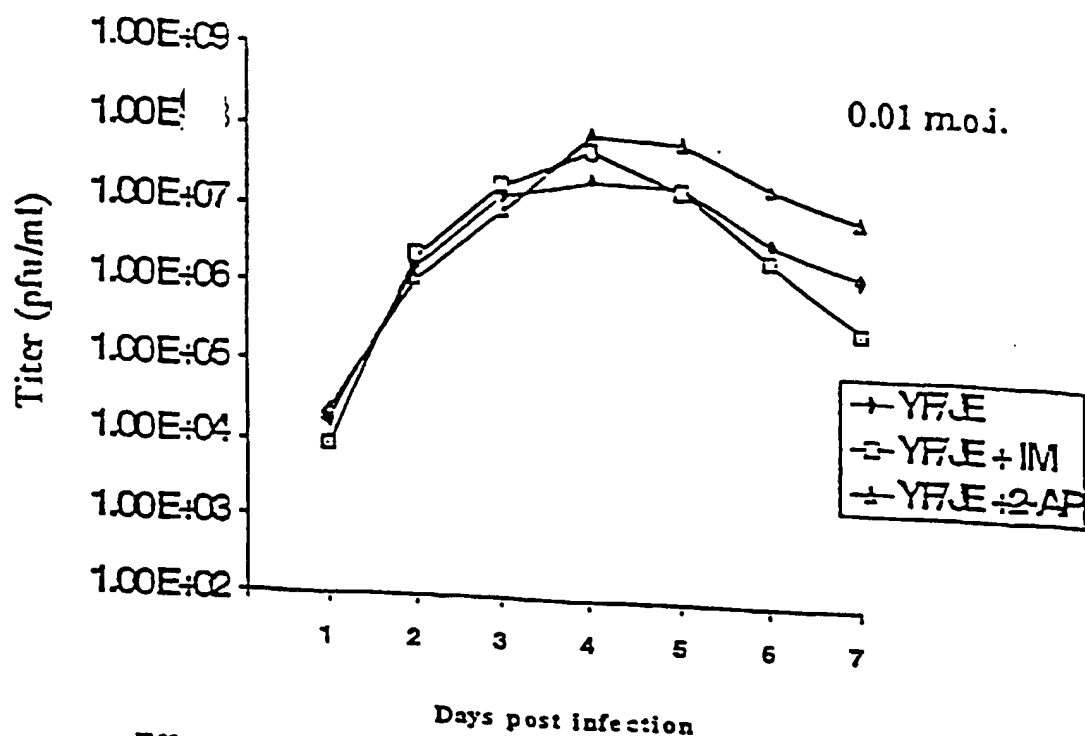
Growth curves of RMS (YF/JE SA14-14-2) in Vero and LLC-MK2 cells.

Fig. 9



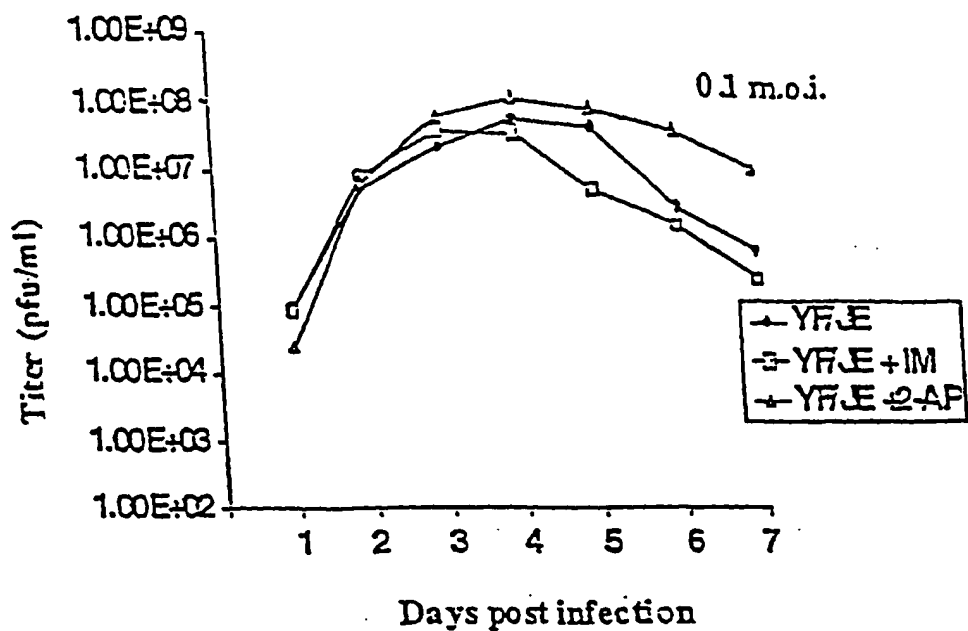
Growth comparison between RMS and YF-Vax in MRC-5 cells.

Fig. 10



YF/JE, Effect of indomethacin (IM) or 2-aminopyrine (2-AP) on growth kinetics of
SA14-14-2 (0.01 MOD) in FRhL cells

Fig. 11A



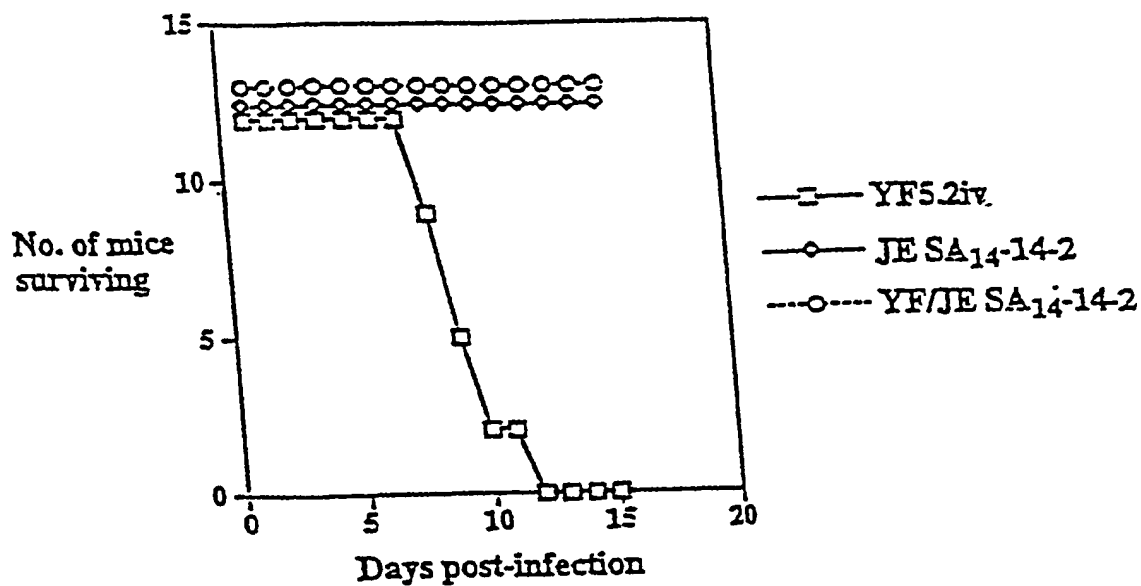
Effect of indomethacin or 2-aminopurine on growth kinetics of YF/JE_{3A1+142} (0.1 MOI) in FRhL cells.

Fig. 11 B

Mouse neurovirulence analysis

MICE: 4 week old ICR males/females

VIRUS DOSE: 10^4 pfu intracerebrally



Virus	Survival	P
YF5.2iv	0/12 (0%)	-
JE SA ₁₄₋₁₄₋₂	12/12 (100%)	<0.001
YF/JE SA ₁₄₋₁₄₋₂	13/13 (100%)	<0.001

Fig. 12

Neutralizing antibody response
to YF/JE SA 14-14-2 chimeric vaccine
(3-week old mice immunized, samples for testing taken at 6 weeks)

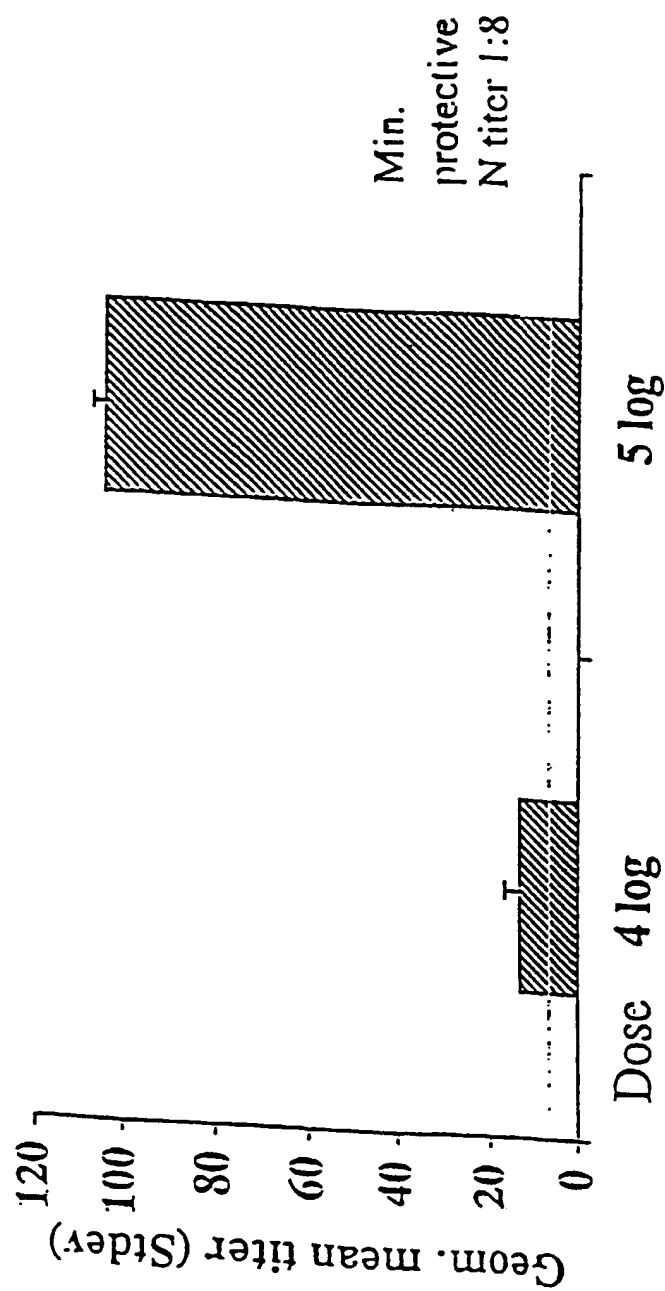


Fig. 13

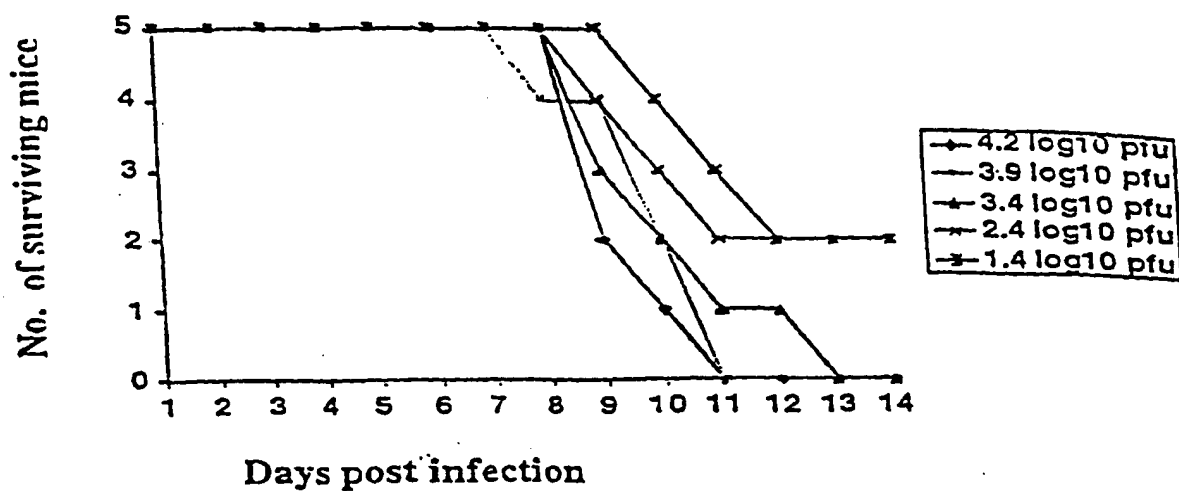


Fig. 14A Neurovirulence testing of YF-Vax in 4-week old ICR mice by the i.c. route.

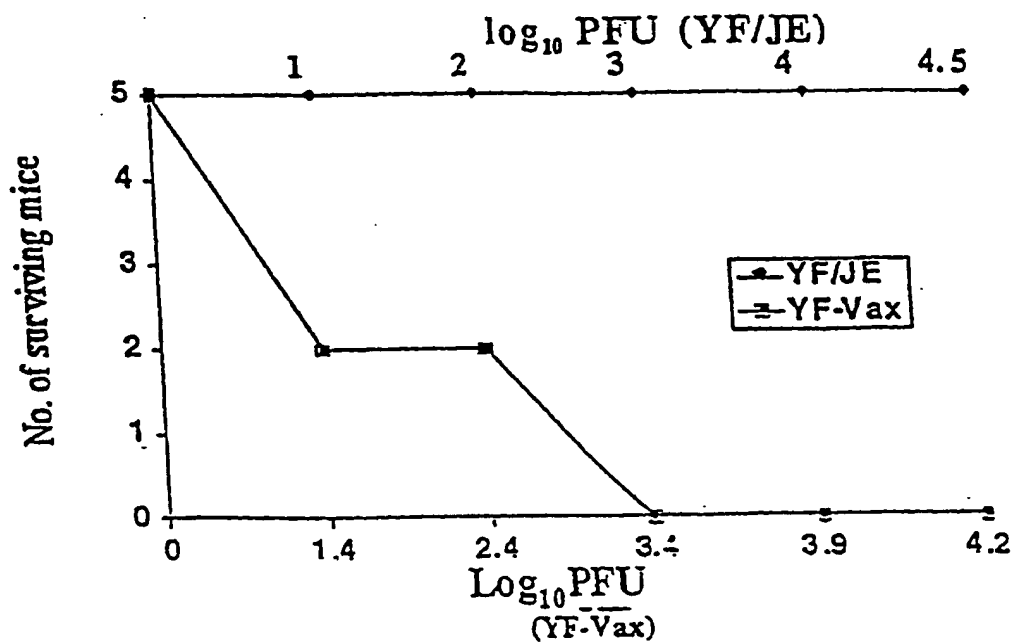


Fig. 14B Neurovirulence testing of YF/JE_{SA14-14-2} in 4-week old ICR mice by I.C. route

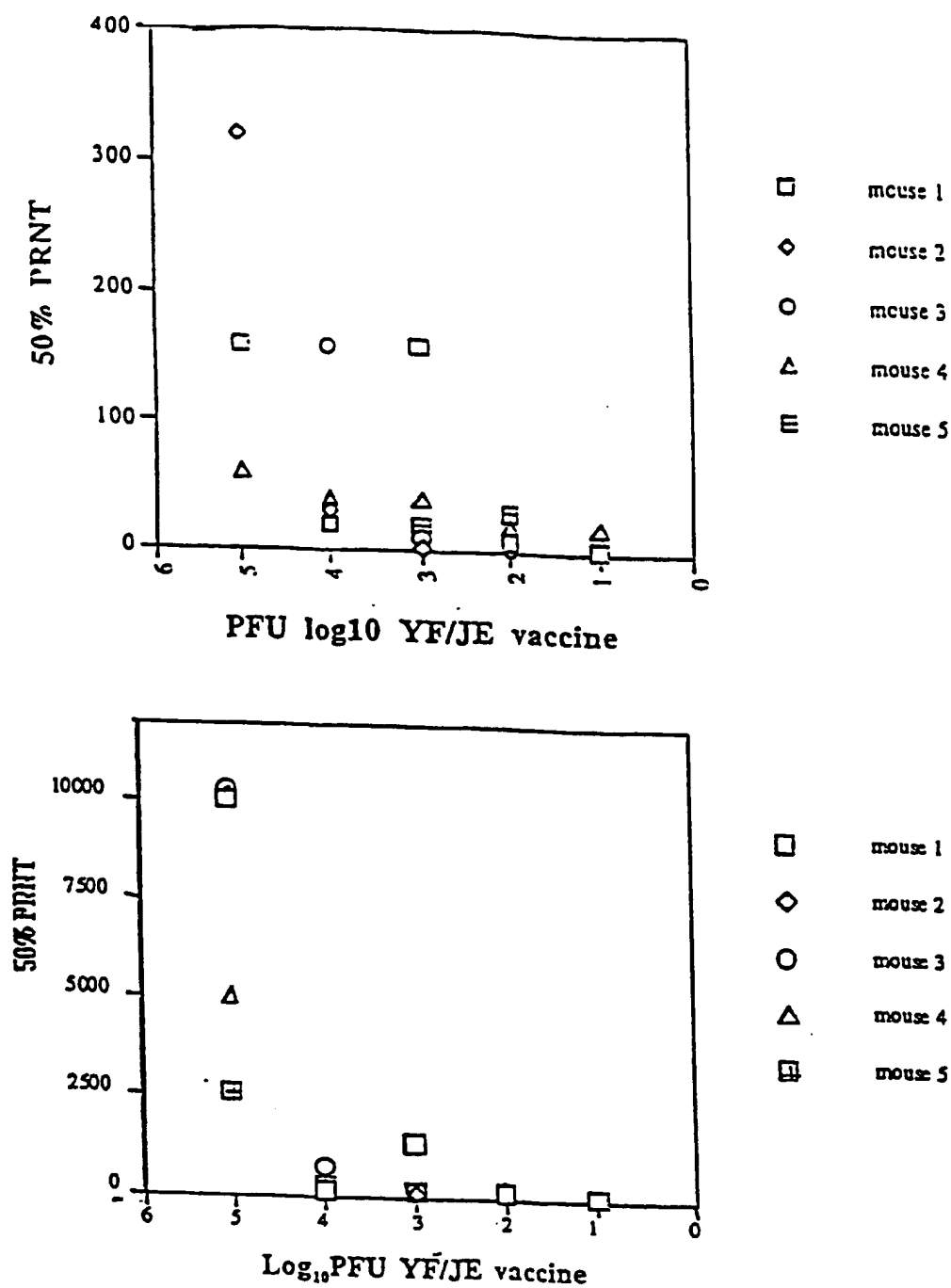


Fig. 15 Neutralizing antibody titers in mice inoculated s.c. with graded doses of YF/JE vaccine. TOP: 3 weeks post immunization and BOTTOM: 8 weeks post immunization

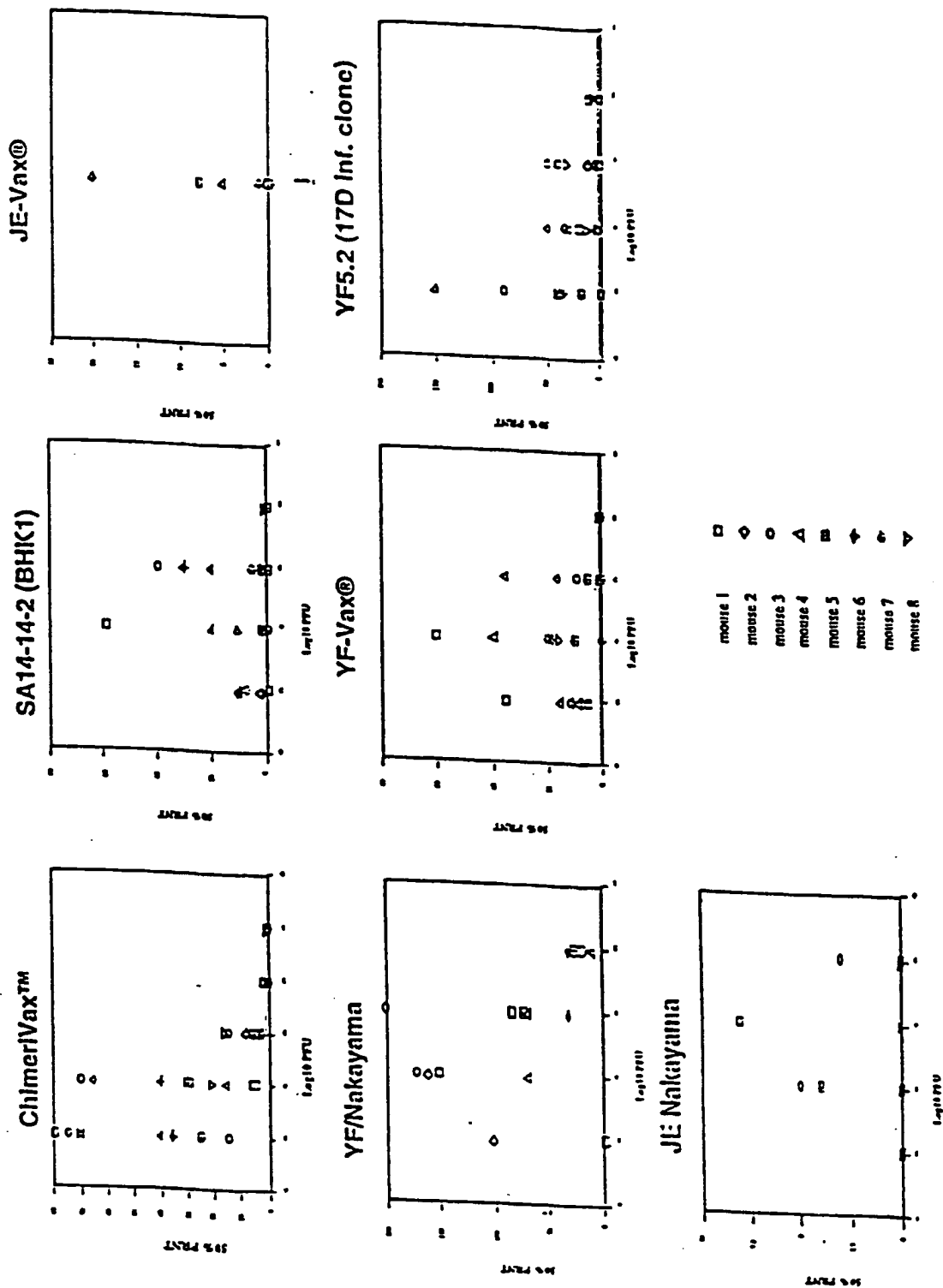
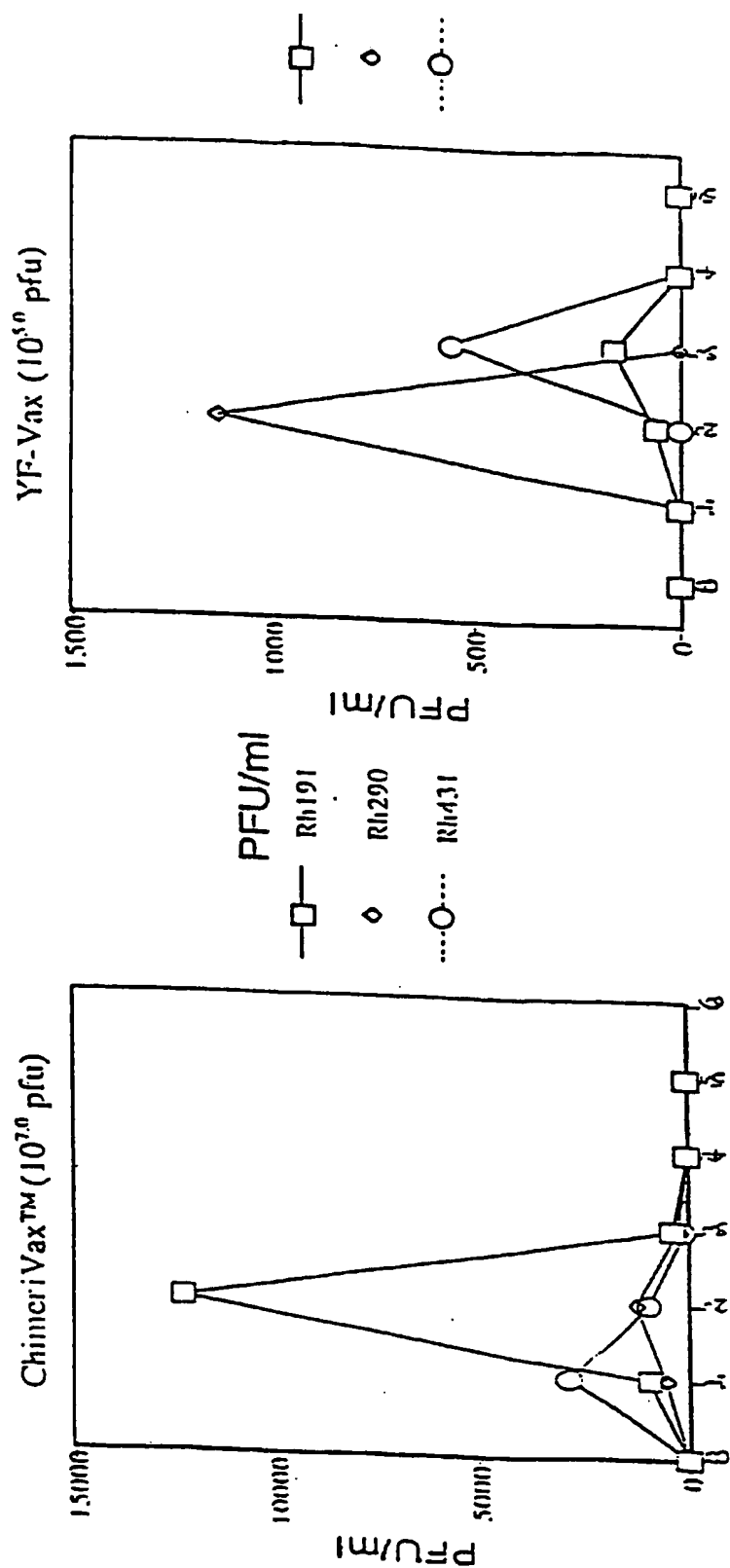
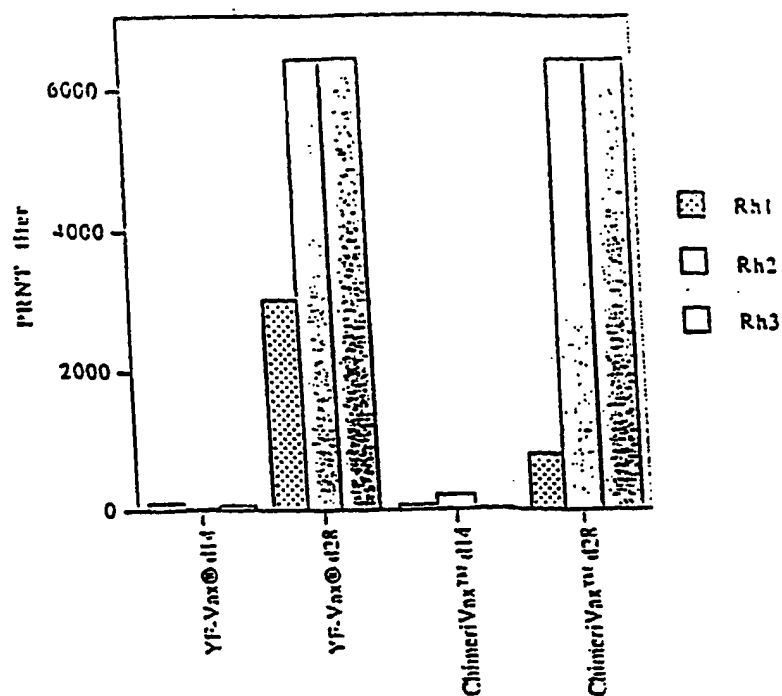


Fig. 16 SEROLOGICAL RESPONSES OF MICE IMMUNIZED WITH A SINGLE DOSE OF LIVE VIRUSES



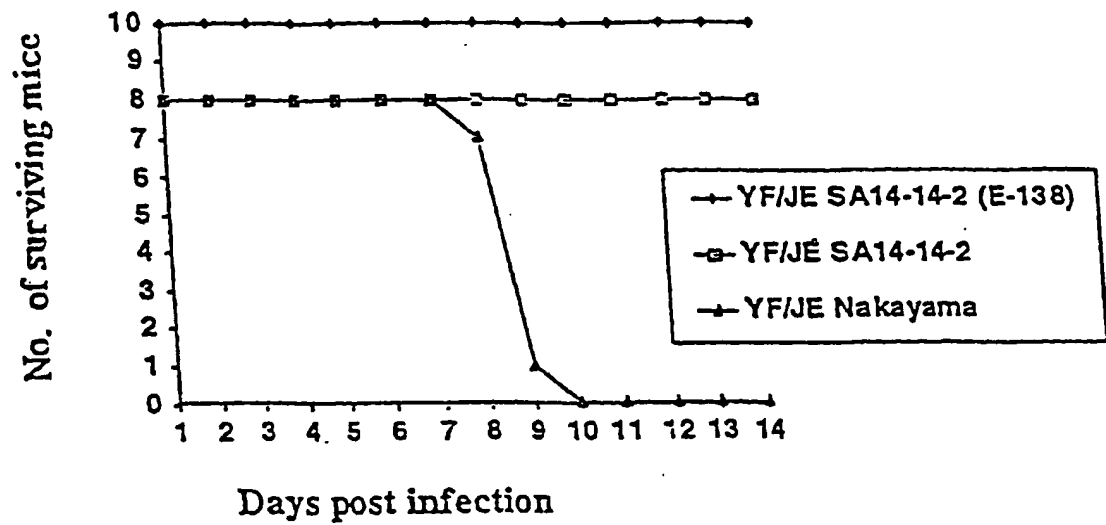
Viremia and GMT of viremia in 3 rhesus monkeys inoculated with ChimeriVax™ or YF-Vax® by the I.C. route.

Fig. 17



Neutralizing antibody titers (50%) in rhesus monkeys 2 and 4 weeks post inoculations with a single dose of vaccines by the I.C. route.

Fig. 18



Mouse neurovirulence testing of YF/JE SA14-14-2 (E-138 K→E) mutant.

Fig. 19

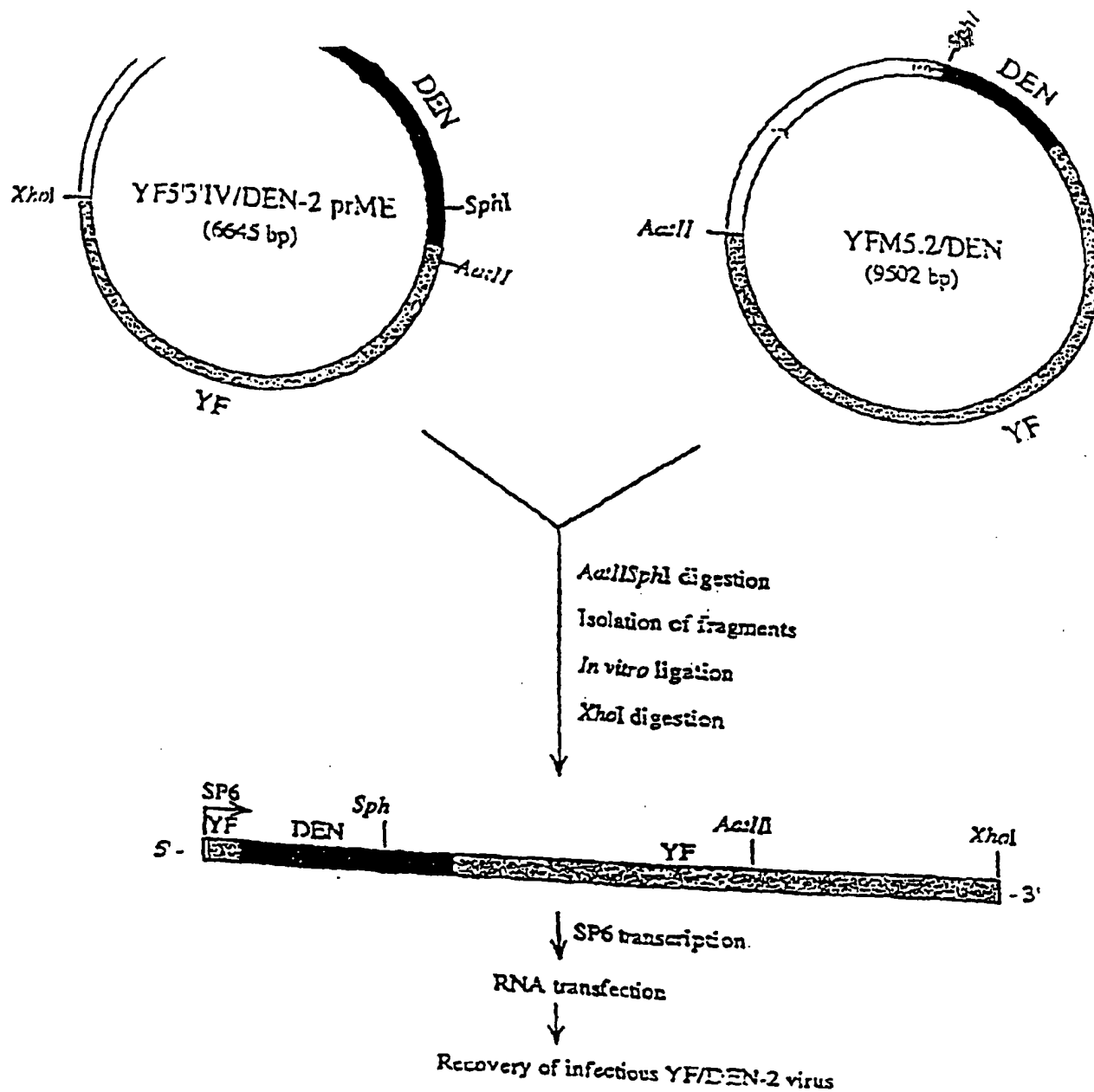


Fig. 20

Structure of modified YF clones expressing
E/NS1 intergenic open reading frames

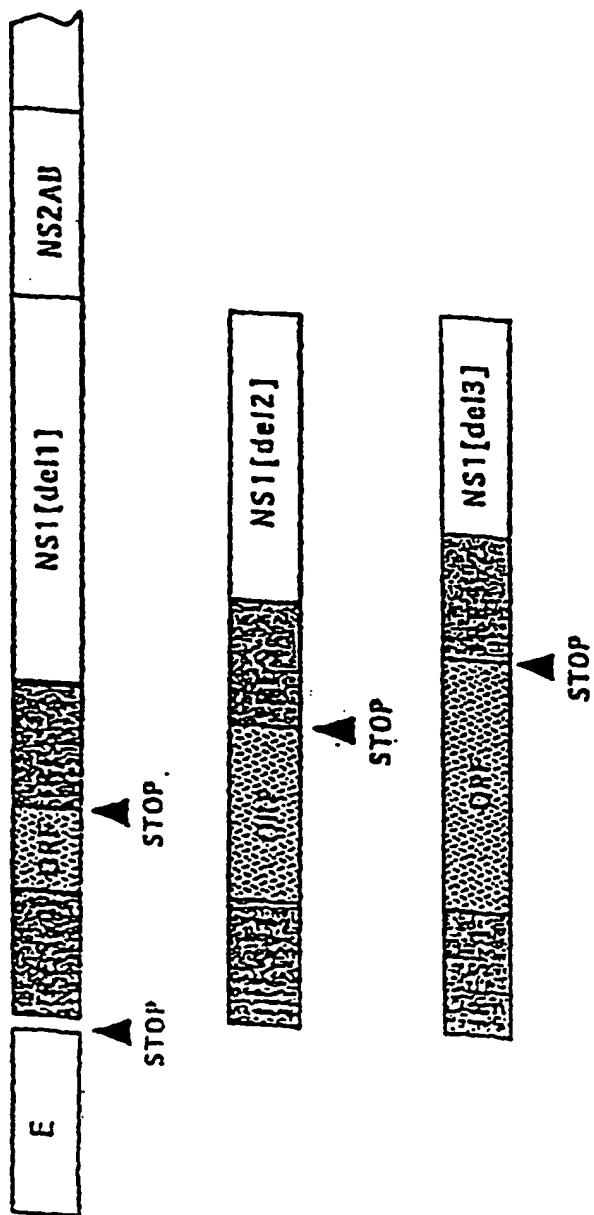
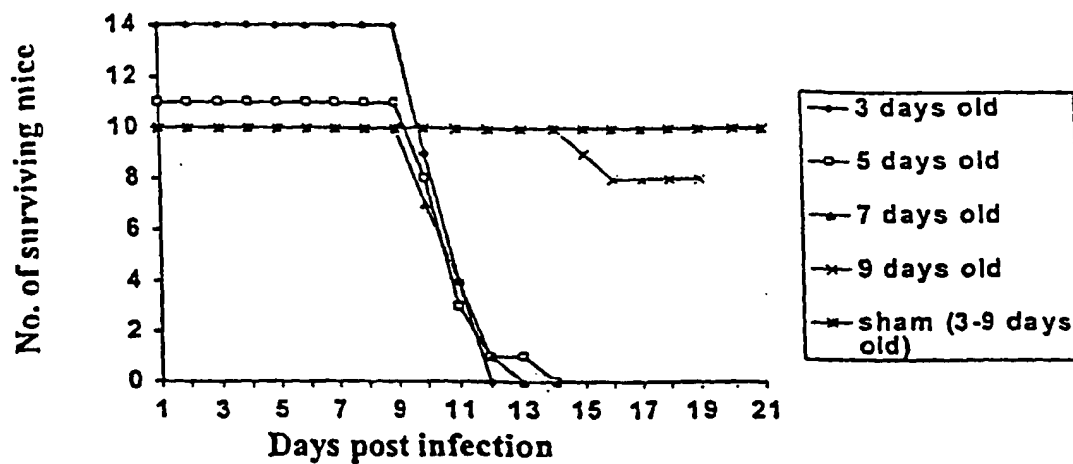
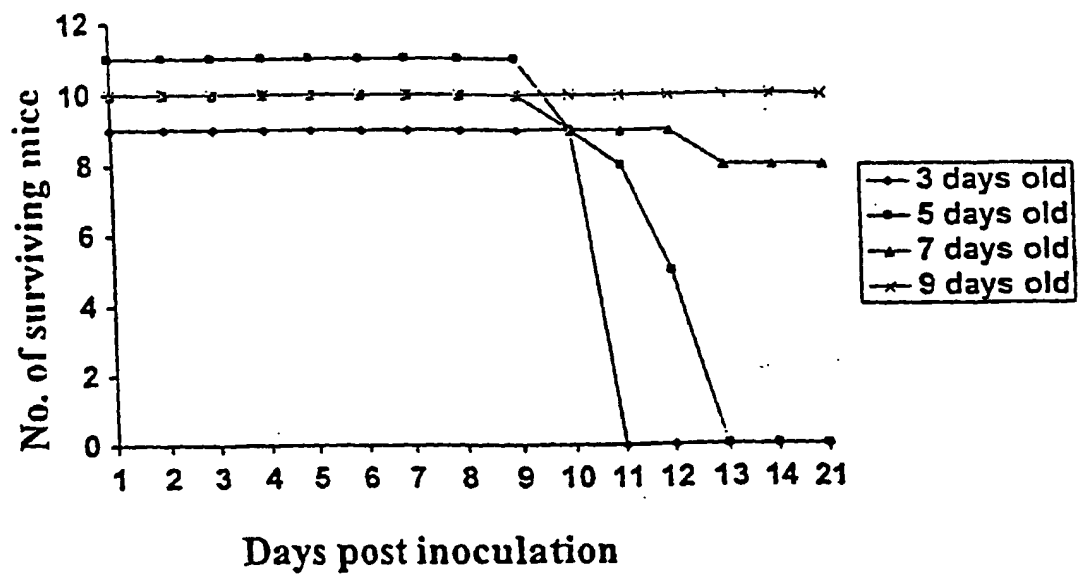


Fig.21



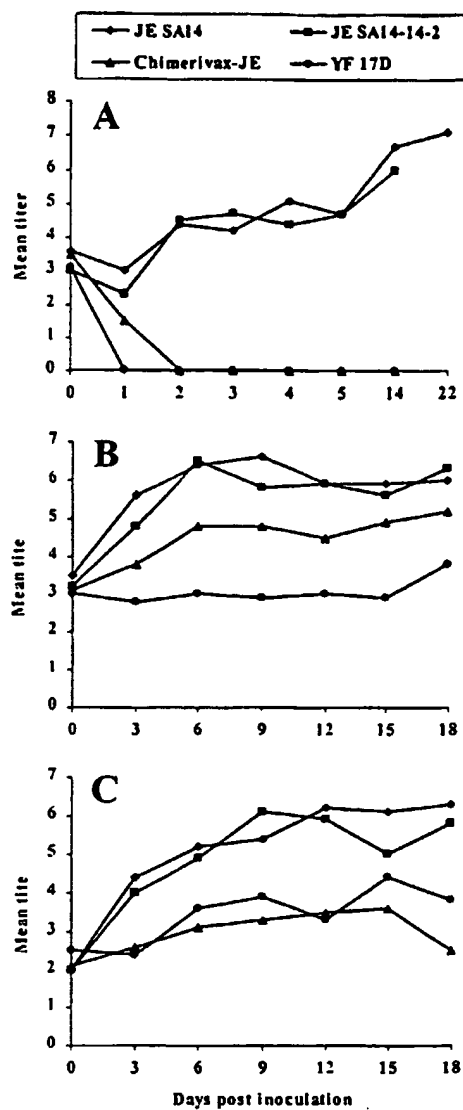
Neurovirulence phenotype of ChimeriVax™-Den2 in outbred (CD-1) suckling mice inoculated by the I.C. route with 10,000 PFU/0.02 ml.

Fig. 22



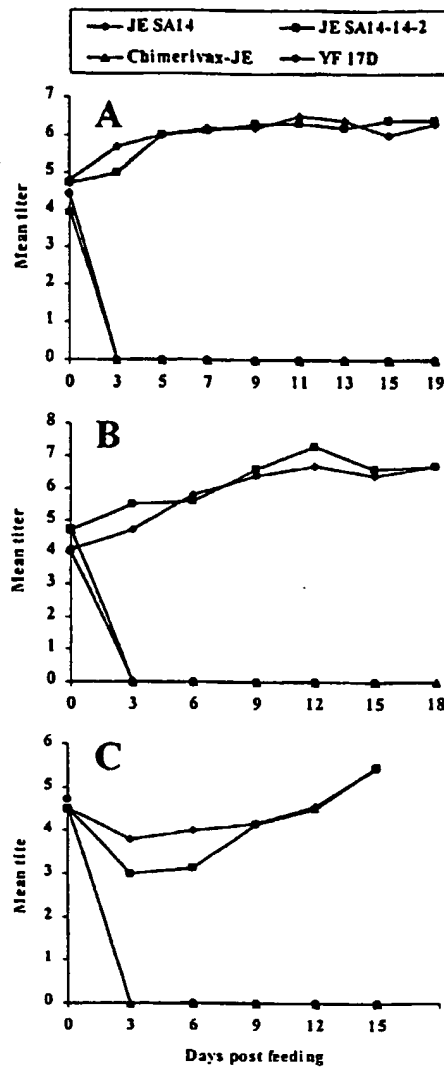
Neurovirulence phenotype of 17D vaccine (YF-Vax®) in outbred (CD-1) suckling mice inoculated by the I.P. route with 1000 PFU/0.02 ml.

Fig. 23



Figs. 24A-C

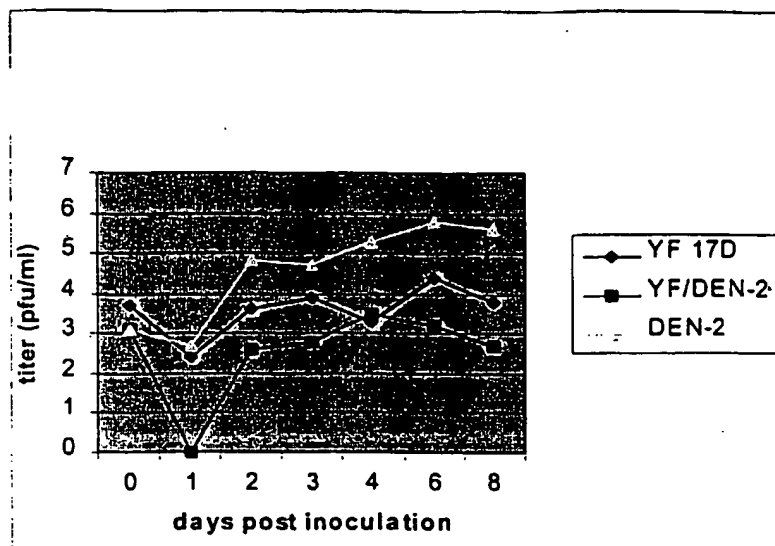
Growth of JE SA14, JE SA14-14-2, ChimeriVaxTM-JE and YF 17D intrathoracically inoculated mosquitoes. A. *Cx. tritaeniorhynchus* mosquitoes, B. *Ae. albopictus* mosquitoes, C. *Ae. aegypti* mosquitoes. Mean titer = geometric mean of the titers of three individual mosquitoes; log₁₀ pfu/mosquito.



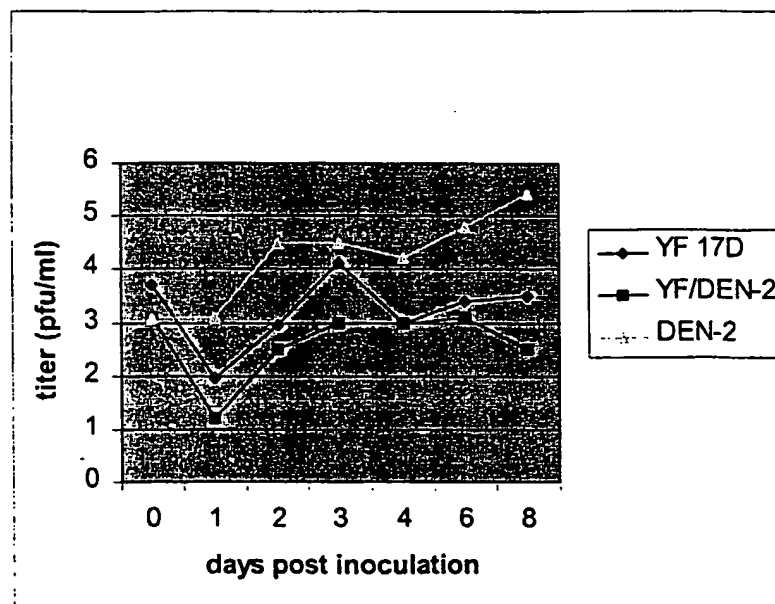
Figs. 25 A-C

Growth of JE SA14, JE SA14-14-2, ChimeriVax™-JE and YF 17D IT orally exposed mosquitoes. A. *Cx. tritaeniorhynchus* mosquitoes, B. *Ae. albopictus* mosquitoes, C. *Ae. aegypti* mosquitoes. Mean titer = geometric mean of the titers of three individual mosquitoes; log₁₀ pfu/mosquito.

A



B



. Growth of virus in IT inoculated *Ae. aegypti* (A) and *Ae. albopictus* (B) mosquitoes.

Figs. 26 A and B

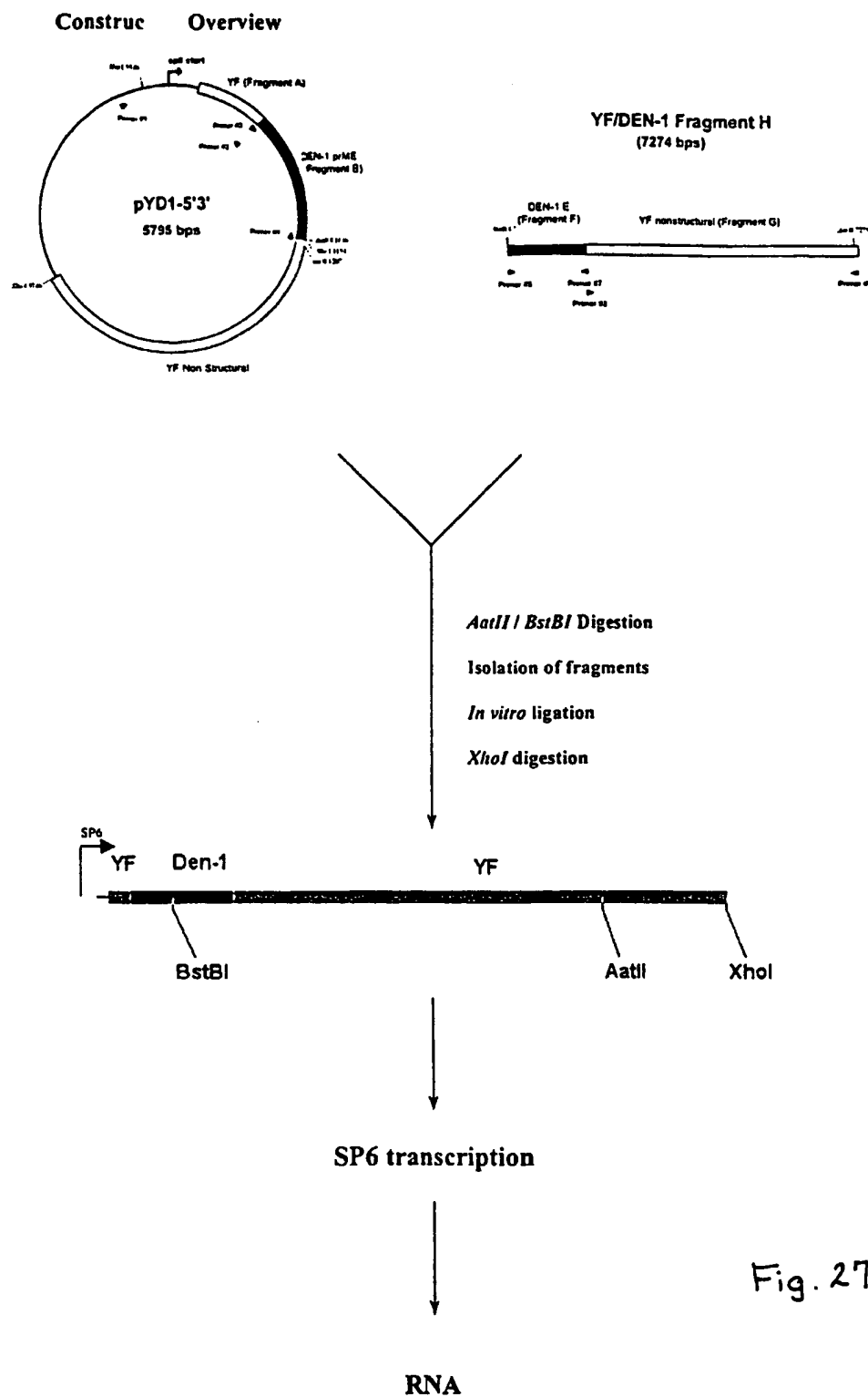


Fig. 27

Plasmid and Fragment Maps

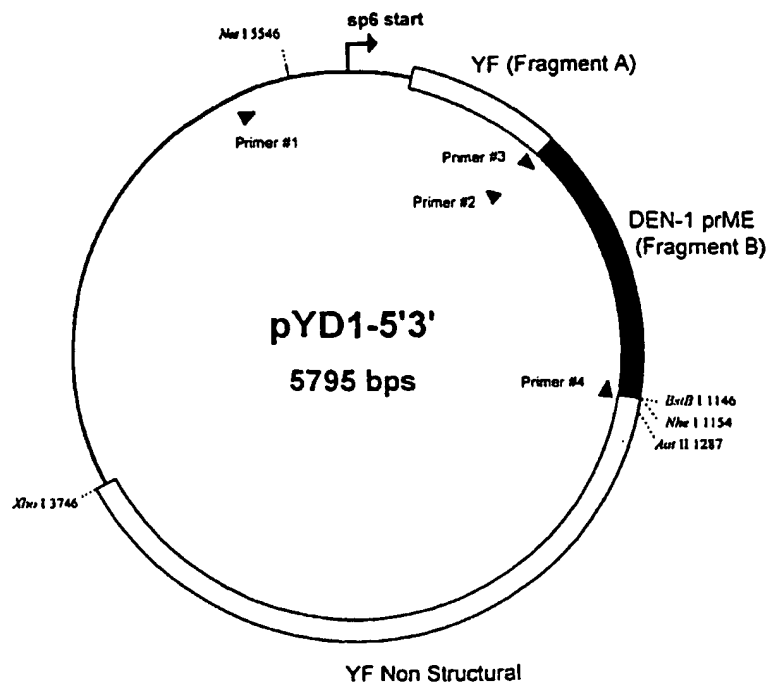
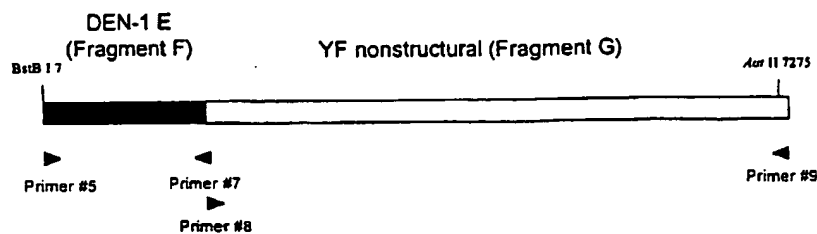
YF/DEN-1 Fragment H
(7274 bps)

Fig. 28

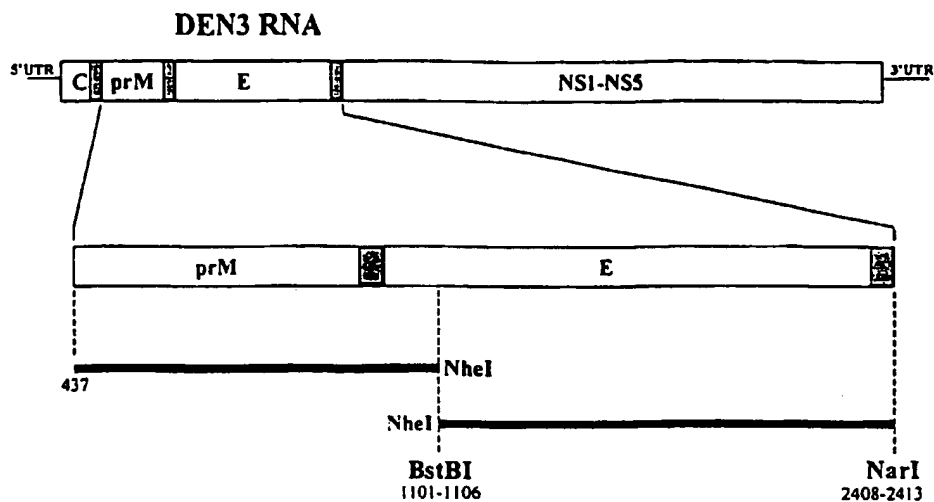


Figure 1. RT-PCR amplification of the prM-E region of the PaH881/88 DEN3 virus genome. The virus genome is shown on the top diagram. Regions encoding hydrophobic signals for corresponding downstream proteins are shadowed. The prM-E region was amplified in two fragments (black solid lines). Restriction sites introduced for subsequent in-frame in vitro ligation into YF backbone (BstBI and NarI) and cloning (NheI) are indicated.

Fig.29

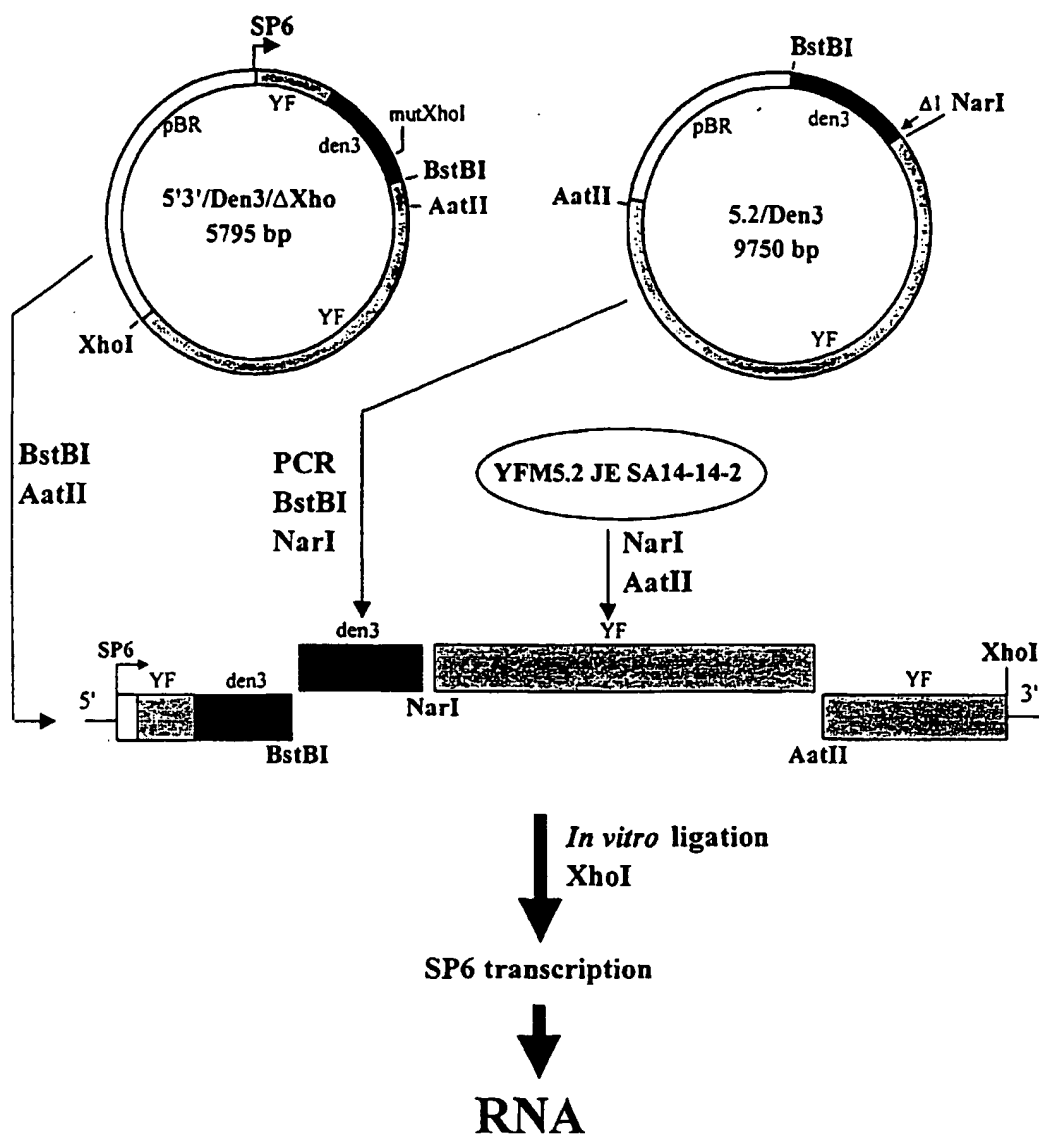


Fig. 30

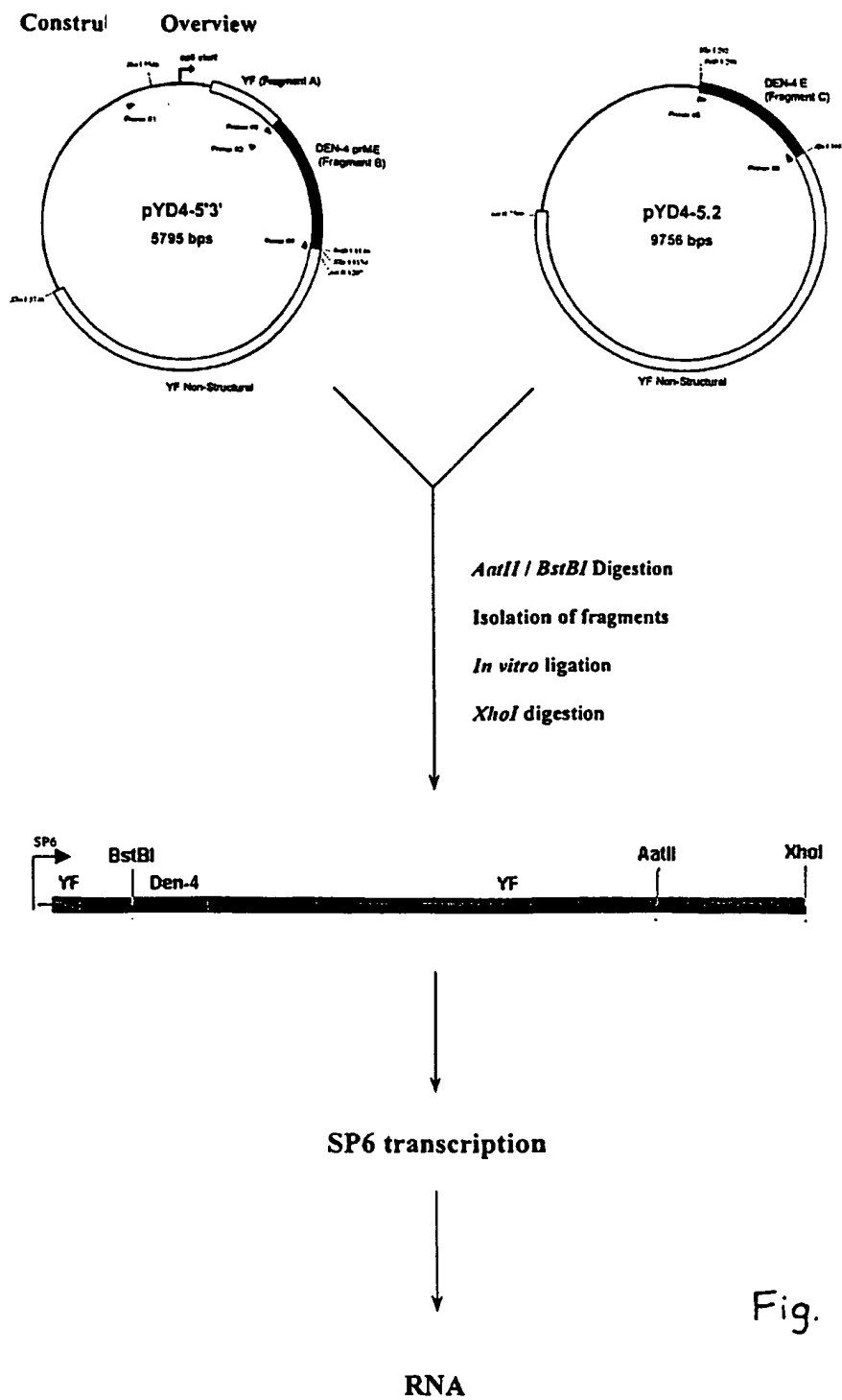


Fig. 31

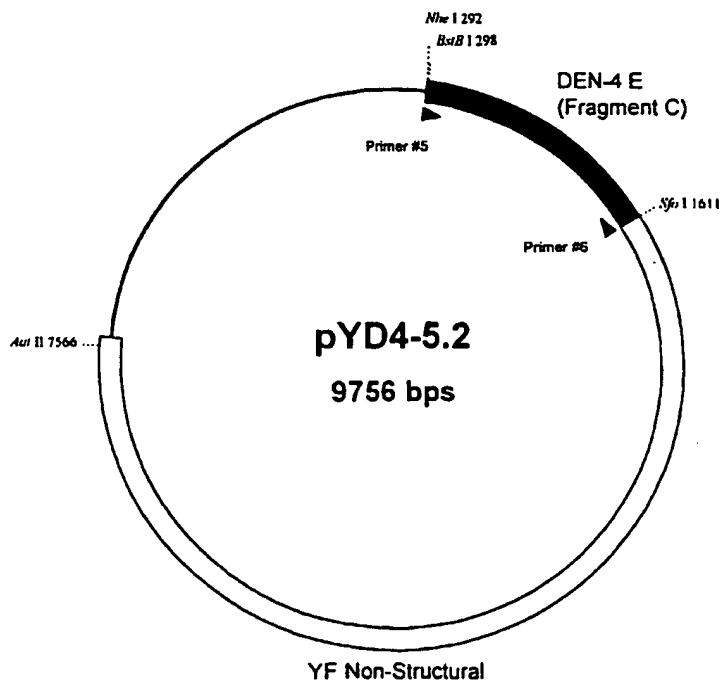
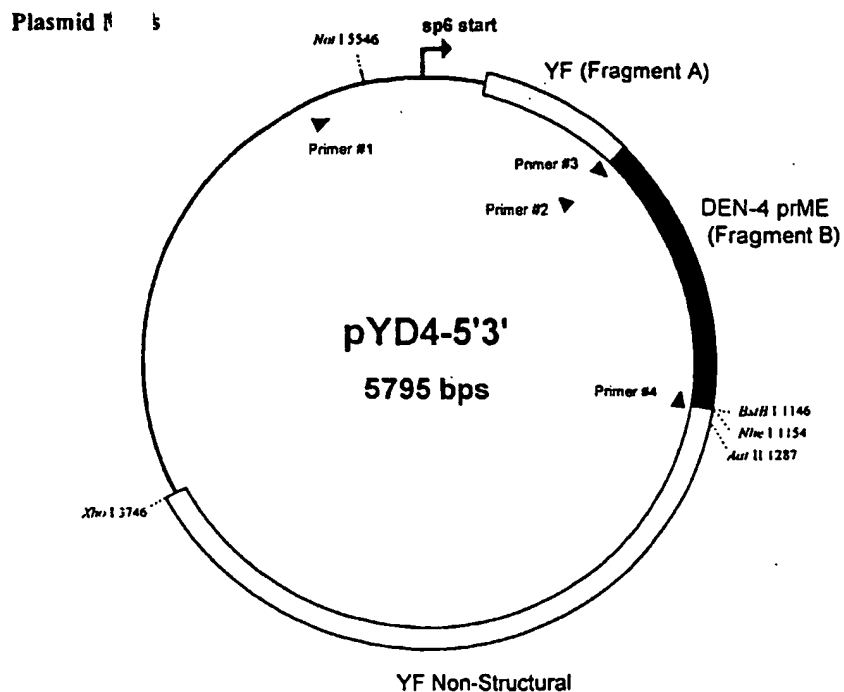


Fig. 32

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<213> Dengue-3 virus

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tacatcgaca tgggtgac 18

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gacatgggga gctaacgc 18

<210> 36
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cccgagggtt ccatattcag g 21

<210> 37
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<212> DNA
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<400> 37
ggaacaggaa agagcttc 18

<210> 38
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<213> Dengue-3 virus

<400> 38
gagtattgtc ccatgctg 18

<210> 39
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<212> DNA
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<400> 39
ggaattggag acaaagcc 18

<210> 40
<211> 21
<212> DNA
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<400> 40
tggatagtgg acagacagtg g 21

<210> 41
<211> 22
<212> DNA
<213> Dengue-3 virus

<400> 41
ctctaaatat gaagatacca tc 22

<210> 42
<211> 39
<212> DNA
<213> Dengue-3 virus

<400> 42
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<210> 43
<211> 27
<212> DNA
<213> Dengue-3 virus

<400> 43
gtgagcattg agaaagcgcc acgcttc 27

<210> 44
<211> 33
<212> DNA
<213> Dengue-3 virus

<400> 44
tccaccgctc atcaacagca ttcccaaat tag 33

<210> 45
<211> 46
<212> DNA
<213> Dengue-3 virus

<400> 45
gaatgctgtt gatgacgggt gaatttcacc tgtcaacaag agacgg 46

<210> 46
<211> 39
<212> DNA
<213> Dengue-3 virus

<400> 46
gccgctagcg gttcgaaata gagccacttc cttggctgt 39

<210> 47
<211> 40
<212> DNA
<213> Dengue-3 virus

<400> 47
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<210> 48
<211> 35
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<213> Dengue-3 virus

<400> 48
gccgccggcg ccaactgtga aacctagaaa cacag 35

<210> 49
<211> 39
<212> DNA
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<400> 49
atttaggtga cactatagag taaatcctgt gtgctaatt 39

<210> 50
<211> 1983

<212> DNA

<213> Dengue-2 virus

<220>

<221> CDS

<222> (1)...(1983)

<400> 50

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Phe His Leu Thr Thr Arg Asn Gly Glu Pro His Met Ile Val Ser Arg
1 5 10 15

caa gag aaa ggg aaa agt ctt ttg ttt aaa aca gag gat ggc gtg aac 96
Gln Glu Lys Gly Lys Ser Leu Leu Phe Lys Thr Glu Asp Gly Val Asn
20 25 30

atg tgc acc ctc atg gcc atg gac ctt ggt gaa ttg tgt gaa gac aca 144
Met Cys Thr Leu Met Ala Met Asp Leu Gly Glu Leu Cys Glu Asp Thr
35 40 45

atc acg tac aag tgt ccc ctt ctc agg cag aat gag cca gaa gac ata 192
Ile Thr Tyr Lys Cys Pro Leu Leu Arg Gln Asn Glu Pro Glu Asp Ile
50 55 60

gac tgc tgg tgc aac tcc acg tcc acg tgg gta acc tat ggg act tgt 240
Asp Cys Trp Cys Asn Ser Thr Ser Thr Trp Val Thr Tyr Gly Thr Cys
65 70 75 80

acc acc acg gga gaa cat aga aga gaa aaa aga tca gtg gca ctc gtt 288
Thr Thr Thr Gly Glu His Arg Arg Glu Lys Arg Ser Val Ala Leu Val
85 90 95

cca cat gtg gga atg gga ctg gag acg cga act gaa aca tgg atg tca 336
Pro His Val Gly Met Gly Leu Glu Thr Arg Thr Glu Thr Trp Met Ser
100 105 110

tca gaa ggg gct tgg aaa cat gcc cag aga att gaa att tgg atc ctg 384
Ser Glu Gly Ala Trp Lys His Ala Gln Arg Ile Glu Ile Trp Ile Leu
115 120 125

aga cat cca ggc ttc acc ata atg gca gca atc ctg gca tac acc ata 432
Arg His Pro Gly Phe Thr Ile Met Ala Ala Ile Leu Ala Tyr Thr Ile
130 135 140

ggg acg aca cat ttc cag aga gca ctg att ttc atc tta ctg aca gct 480
Gly Thr Thr His Phe Gln Arg Ala Leu Ile Phe Ile Leu Leu Thr Ala
145 150 155 160

gtc gct cct tca atg aca atg cgt tgc ata gga ata tca aat aga gac 528
Val Ala Pro Ser Met Thr Met Arg Cys Ile Gly Ile Ser Asn Arg Asp
165 170 175

ttt gta gaa ggg gtt tca gga gga agc tgg gtt gac ata gtc tta gaa 576
Phe Val Glu Gly Val Ser Gly Gly Ser Trp Val Asp Ile Val Leu Glu
180 185 190

cat gga agc tgt gtg acg acg atg gca aaa aac aaa cca aca ttg gat 624
His Gly Ser Cys Val Thr Thr Met Ala Lys Asn Lys Pro Thr Leu Asp

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195	200	205	
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tac tgt ata gag gca aag cta acc aac aca aca gaa tct cgt tgc Tyr Cys Ile Glu Ala Lys Leu Thr Asn Thr Thr Glu Ser Arg Cys 225 230 235 240			720
cca aca caa ggg gaa ccc agc cta aat gaa gag cag gat aaa agg ttc Pro Thr Gln Gly Glu Pro Ser Leu Asn Glu Glu Gln Asp Lys Arg Phe 245 250 255			768
gtc tgc aaa cac tcc atg gta gac aga gga tgg gga aat gga tgt gga Val Cys Lys His Ser Met Val Asp Arg Gly Trp Gly Asn Gly Cys Gly 260 265 270			816
tta ttt gga aag gga ggc att gtg acc tgt gct atg ttc aca tgc aaa Leu Phe Gly Lys Gly Gly Ile Val Thr Cys Ala Met Phe Thr Cys Lys 275 280 285			864
aag aac atg gag gga aaa gtt gtg cag cca gaa aac ttg gaa tac acc Lys Asn Met Glu Gly Lys Val Val Gln Pro Glu Asn Leu Glu Tyr Thr 290 295 300			912
att gtg gta aca ccc cac tca ggg gaa gag cat gcg gtc gga aat gac Ile Val Val Thr Pro His Ser Gly Glu Glu His Ala Val Gly Asn Asp 305 310 315 320			960
aca gga aaa cat ggc aag gaa atc aaa gta aca cca cag agt tcc atc Thr Gly Lys His Gly Lys Glu Ile Lys Val Thr Pro Gln Ser Ser Ile 325 330 335			1008
aca gaa gca gaa ttg aca ggt tat ggc act gtc acg atg gag tgc tct Thr Glu Ala Glu Leu Thr Gly Tyr Gly Thr Val Thr Met Glu Cys Ser 340 345 350			1056
ccg aga aca ggc ctc gac ttc aat gag atg gtg ttg ctg cag atg gaa Pro Arg Thr Gly Leu Asp Phe Asn Glu Met Val Leu Leu Gln Met Glu 355 360 365			1104
aat aaa gct tgg ctg gtg cat agg caa tgg ttc cta gac ctg ccg tta Asn Lys Ala Trp Leu Val His Arg Gln Trp Phe Leu Asp Leu Pro Leu 370 375 380			1152
cca tgg ctg ccc gga gcg gac aca caa ggg tca aat tgg ata caa aaa Pro Trp Leu Pro Gly Ala Asp Thr Gln Gly Ser Asn Trp Ile Gln Lys 385 390 395 400			1200
gaa aca ttg gtc act ttc aaa aat cct cat gcg aag aaa cag gat gtt Glu Thr Leu Val Thr Phe Lys Asn Pro His Ala Lys Lys Gln Asp Val 405 410 415			1248
gtt gtt tta gga tcc caa gaa ggg gcc atg cac aca gca ctc aca ggg Val Val Leu Gly Ser Gln Glu Gly Ala Met His Thr Ala Leu Thr Gly 420 425 430			1296

gcc aca gaa atc caa atg tca tca gga aac tta ctc ttc aca gga cat	1344
Ala Thr Glu Ile Gln Met Ser Ser Gly Asn Leu Leu Phe Thr Gly His	
435 440 445	
ctc aag tgc agg ctg aga atg gac aag cta cag ctc aaa gga atg tca	1392
Leu Lys Cys Arg Leu Arg Met Asp Lys Leu Gln Leu Lys Gly Met Ser	
450 455 460	
tac tct atg tgc aca gga aag ttt aaa gtt gtg aag gaa ata gca gaa	1440
Tyr Ser Met Cys Thr Gly Lys Phe Lys Val Val Lys Glu Ile Ala Glu	
465 470 475 480	
aca caa cat gga aca ata gtt atc agg gtg cag tat gaa ggg gac ggc	1488
Thr Gln His Gly Thr Ile Val Ile Arg Val Gln Tyr Glu Gly Asp Gly	
485 490 495	
tct cca tgt aaa atc cct ttt gag ata atg gat ttg gaa aaa aga cat	1536
Ser Pro Cys Lys Ile Pro Phe Glu Ile Met Asp Leu Glu Lys Arg His	
500 505 510	
gtc tta ggt cgc ctg atc aca gtc aac cca att gtg aca gaa aaa gat	1584
Val Leu Gly Arg Leu Ile Thr Val Asn Pro Ile Val Thr Glu Lys Asp	
515 520 525	
agc cca gtc aac ata gaa gca gaa cct cca ttc gga gac agc tac atc	1632
Ser Pro Val Asn Ile Glu Ala Glu Pro Pro Phe Gly Asp Ser Tyr Ile	
530 535 540	
atc ata gga gta gag ccg gga caa ctg aag ctc aac tgg ttt aag aaa	1680
Ile Ile Gly Val Glu Pro Gly Gln Leu Lys Leu Asn Trp Phe Lys Lys	
545 550 555 560	
gga agt tct atc ggc caa atg ttt gag aca aca atg agg ggg gcg aag	1728
Gly Ser Ser Ile Gly Gln Met Phe Glu Thr Thr Met Arg Gly Ala Lys	
565 570 575	
aga atg gcc att ttg ggt gac aca gcc tgg gat ttt gga tcc ctg gga	1776
Arg Met Ala Ile Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Leu Gly	
580 585 590	
gga gtg ttt aca tct ata gga aaa gcc ctc cac caa gtc ttt gga gca	1824
Gly Val Phe Thr Ser Ile Gly Lys Ala Leu His Gln Val Phe Gly Ala	
595 600 605	
atc tat gga gct gcc ttc agt ggg gtc tca tgg act atg aaa atc ctc	1872
Ile Tyr Gly Ala Ala Phe Ser Gly Val Ser Trp Thr Met Lys Ile Leu	
610 615 620	
ata gga gtc att atc aca tgg ata gga atg aat tca cgc agc acc tca	1920
Ile Gly Val Ile Ile Thr Trp Ile Gly Met Asn Ser Arg Ser Thr Ser	
625 630 635 640	
ctg tct gtg tca cta gta ttg gtg gga gtc gtg acg ctg tat ttg gga	1968
Leu Ser Val Ser Leu Val Leu Val Gly Val Val Thr Leu Tyr Leu Gly	
645 650 655	
gtt atg gtg ggc gcc	1983
Val Met Val Gly Ala	

660

<210> 51
 <211> 661
 <212> PRT
 <213> Dengue-2 virus

<400> 51
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 35 40 45
 Ile Thr Tyr Lys Cys Pro Leu Leu Arg Gln Asn Glu Pro Glu Asp Ile
 50 55 60
 Asp Cys Trp Cys Asn Ser Thr Ser Thr Trp Val Thr Tyr Gly Thr Cys
 65 70 75 80
 Thr Thr Thr Gly Glu His Arg Arg Glu Lys Arg Ser Val Ala Leu Val
 85 90 95
 Pro His Val Gly Met Gly Leu Glu Thr Arg Thr Glu Thr Trp Met Ser
 100 105 110
 Ser Glu Gly Ala Trp Lys His Ala Gln Arg Ile Glu Ile Trp Ile Leu
 115 120 125
 Arg His Pro Gly Phe Thr Ile Met Ala Ala Ile Leu Ala Tyr Thr Ile
 130 135 140
 Gly Thr Thr His Phe Gln Arg Ala Leu Ile Phe Ile Leu Leu Thr Ala
 145 150 155 160
 Val Ala Pro Ser Met Thr Met Arg Cys Ile Gly Ile Ser Asn Arg Asp
 165 170 175
 Phe Val Glu Gly Val Ser Gly Gly Ser Trp Val Asp Ile Val Leu Glu
 180 185 190
 His Gly Ser Cys Val Thr Thr Met Ala Lys Asn Lys Pro Thr Leu Asp
 195 200 205
 Phe Glu Leu Ile Lys Thr Glu Ala Lys Gln Pro Ala Thr Leu Arg Lys
 210 215 220
 Tyr Cys Ile Glu Ala Lys Leu Thr Asn Thr Thr Thr Glu Ser Arg Cys
 225 230 235 240
 Pro Thr Gln Gly Glu Pro Ser Leu Asn Glu Glu Gln Asp Lys Arg Phe
 245 250 255
 Val Cys Lys His Ser Met Val Asp Arg Gly Trp Gly Asn Gly Cys Gly
 260 265 270
 Leu Phe Gly Lys Gly Gly Ile Val Thr Cys Ala Met Phe Thr Cys Lys
 275 280 285
 Lys Asn Met Glu Gly Lys Val Val Gln Pro Glu Asn Leu Glu Tyr Thr
 290 295 300
 Ile Val Val Thr Pro His Ser Gly Glu Glu His Ala Val Gly Asn Asp
 305 310 315 320
 Thr Gly Lys His Gly Lys Glu Ile Lys Val Thr Pro Gln Ser Ser Ile
 325 330 335
 Thr Glu Ala Glu Leu Thr Gly Tyr Gly Thr Val Thr Met Glu Cys Ser
 340 345 350
 Pro Arg Thr Gly Leu Asp Phe Asn Glu Met Val Leu Leu Gln Met Glu
 355 360 365
 Asn Lys Ala Trp Leu Val His Arg Gln Trp Phe Leu Asp Leu Pro Leu
 370 375 380
 Pro Trp Leu Pro Gly Ala Asp Thr Gln Gly Ser Asn Trp Ile Gln Lys

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385          390          395          400
Glu Thr Leu Val Thr Phe Lys Asn Pro His Ala Lys Lys Gln Asp Val
405          410          415
Val Val Leu Gly Ser Gln Glu Gly Ala Met His Thr Ala Leu Thr Gly
420          425          430
Ala Thr Glu Ile Gln Met Ser Ser Gly Asn Leu Leu Phe Thr Gly His
435          440          445
Leu Lys Cys Arg Leu Arg Met Asp Lys Leu Gln Leu Lys Gly Met Ser
450          455          460
Tyr Ser Met Cys Thr Gly Lys Phe Lys Val Val Lys Glu Ile Ala Glu
465          470          475
Thr Gln His Gly Thr Ile Val Ile Arg Val Gln Tyr Glu Gly Asp Gly
485          490          495
Ser Pro Cys Lys Ile Pro Phe Glu Ile Met Asp Leu Glu Lys Arg His
500          505          510
Val Leu Gly Arg Leu Ile Thr Val Asn Pro Ile Val Thr Glu Lys Asp
515          520          525
Ser Pro Val Asn Ile Glu Ala Glu Pro Pro Phe Gly Asp Ser Tyr Ile
530          535          540
Ile Ile Gly Val Glu Pro Gly Gln Leu Lys Leu Asn Trp Phe Lys Lys
545          550          555
Gly Ser Ser Ile Gly Gln Met Phe Glu Thr Thr Met Arg Gly Ala Lys
565          570          575
Arg Met Ala Ile Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Leu Gly
580          585          590
Gly Val Phe Thr Ser Ile Gly Lys Ala Leu His Gln Val Phe Gly Ala
595          600          605
Ile Tyr Gly Ala Ala Phe Ser Gly Val Ser Trp Thr Met Lys Ile Leu
610          615          620
Ile Gly Val Ile Ile Thr Trp Ile Gly Met Asn Ser Arg Ser Thr Ser
625          630          635
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Val Met Val Gly Ala
660

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<210> 52

<211> 10892

<212> DNA

<213> Artificial Sequence

<220>

<223> derived from Yellow Fever virus and Japanese Encephalitis virus

<221> CDS

<222> (119)...(10381)

<400> 52

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acacatttggg attaatTTta atcgttcggtt gagcgattag cagagaactg accagaac 118
atg tct ggt cgt aaa gct cag gga aaa acc ctg ggc gtc aat atg gta 166
Met Ser Gly Arg Lys Ala Gln Gly Lys Thr Leu Gly Val Asn Met Val
1          5          10          15

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cga cga gga gtt cgc tcc ttg tca aac aaa ata aaa caa aaa aca aaa 214
Arg Arg Gly Val Arg Ser Leu Ser Asn Lys Ile Lys Gln Lys Thr Lys

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20										25										30										
caa att gga aac aga cct gga cct tca aga ggt gtt caa gga ttt atc	262																													
Gln Ile Gly Asn Arg Pro Gly Pro Ser Arg Gly Val Gln Gly Phe Ile																														
35 40 45																														
ttt ttc ttt ttg ttc aac att ttg act gga aaa aag atc aca gcc cac	310																													
Phe Phe Phe Leu Phe Asn Ile Leu Thr Gly Lys Lys Ile Thr Ala His																														
50 55 60																														
cta aag agg ttg tgg aaa atg ctg gac cca aga caa ggc ttg gct gtt	358																													
Leu Lys Arg Leu Trp Lys Met Leu Asp Pro Arg Gln Gly Leu Ala Val																														
65 70 75 80																														
cta agg aaa gtc aag aga gtg gtg gcc agt ttg atg aga gga ttg tcc	406																													
Leu Arg Lys Val Lys Arg Val Val Ala Ser Leu Met Arg Gly Leu Ser																														
85 90 95																														
tca agg aaa cgc cgt tcc cat gat gtt ctg act gtg caa ttc cta att	454																													
Ser Arg Lys Arg Arg Ser His Asp Val Leu Thr Val Gln Phe Leu Ile																														
100 105 110																														
ttg gga atg ctg ttg atg acg ggt gga atg aag ttg tcg aat ttc cag	502																													
Leu Gly Met Leu Leu Met Thr Gly Gly Met Lys Leu Ser Asn Phe Gln																														
115 120 125																														
ggg aag ctt ttg atg acc atc aac aac acg gac att gca gac gtt atc	550																													
Gly Lys Leu Leu Met Thr Ile Asn Asn Thr Asp Ile Ala Asp Val Ile																														
130 135 140																														
gtg att ccc acc tca aaa gga gag aac aga tgt tgg gtt cgg gca atc	598																													
Val Ile Pro Thr Ser Lys Gly Glu Asn Arg Cys Trp Val Arg Ala Ile																														
145 150 155 160																														
gac gtc ggc tac atg tgt gag gac act atc acg tac gaa tgt cct aag	646																													
Asp Val Gly Tyr Met Cys Glu Asp Thr Ile Thr Tyr Glu Cys Pro Lys																														
165 170 175																														
ctt acc atg ggc aat gat cca gag gat gtg gat tgc tgg tgt gac aac	694																													
Leu Thr Met Gly Asn Asp Pro Glu Asp Val Asp Cys Trp Cys Asp Asn																														
180 185 190																														
caa gaa gtc tac gtc caa tat gga cgg tgc acg cgg acc agg cat tcc	742																													
Gln Glu Val Tyr Val Gln Tyr Gly Arg Cys Thr Arg Thr Arg His Ser																														
195 200 205																														
aag cga agc agg aga tcc gtg tcg gtc caa aca cat ggg gag agt tca	790																													
Lys Arg Ser Arg Arg Ser Val Ser Val Gln Thr His Gly Glu Ser Ser																														
210 215 220																														
cta gtg aat aaa aaa gag gct tgg ctg gat tca acg aaa gcc aca cga	838																													
Leu Val Asn Lys Lys Glu Ala Trp Leu Asp Ser Thr Lys Ala Thr Arg																														
225 230 235 240																														
tat ctc atg aaa act gag aac tgg atc ata agg aat cct ggc tat gct	886																													
Tyr Leu Met Lys Thr Glu Asn Trp Ile Ile Arg Asn Pro Gly Tyr Ala																														
245 250 255																														

ttc ctg gcg gcg gta ctt ggc tgg atg ctt ggc agt aac aac ggt caa	934
Phe Leu Ala Ala Val Leu Gly Trp Met Leu Gly Ser Asn Asn Gly Gln	
260 265 270	
cgc gtg gta ttt acc atc ctc ctg ctg ttg gtc gct ccg gct tac agt	982
Arg Val Val Phe Thr Ile Leu Leu Leu Leu Val Ala Pro Ala Tyr Ser	
275 280 285	
ttt aat tgt ctg gga atg ggc aat cgt gac ttc ata gaa gga gcc agt	1030
Phe Asn Cys Leu Gly Met Gly Asn Arg Asp Phe Ile Glu Gly Ala Ser	
290 295 300	
ggg gcc act tgg gtg gac ttg gtg cta gaa gga gac agc tgc ttg aca	1078
Gly Ala Thr Trp Val Asp Leu Val Leu Glu Gly Asp Ser Cys Leu Thr	
305 310 315 320	
atc atg gca aac gac aaa cca aca ttg gac gtc cgc atg att aac atc	1126
Ile Met Ala Asn Asp Lys Pro Thr Leu Asp Val Arg Met Ile Asn Ile	
325 330 335	
gaa gct agc caa ctt gct gag gtc aga agt tac tgc tat cat gct tca	1174
Glu Ala Ser Gln Leu Ala Glu Val Arg Ser Tyr Cys Tyr His Ala Ser	
340 345 350	
gtc act gac atc tcg acg gtg gct cgg tgc ccc acg act gga gaa gcc	1222
Val Thr Asp Ile Ser Thr Val Ala Arg Cys Pro Thr Thr Gly Glu Ala	
355 360 365	
cac aac gag aag cga gct gat agt agc tat gtg tgc aaa caa ggc ttc	1270
His Asn Glu Lys Arg Ala Asp Ser Ser Tyr Val Cys Lys Gln Gly Phe	
370 375 380	
act gac cgt ggg tgg ggc aac gga tgt gga ttt ttc ggg aag gga agc	1318
Thr Asp Arg Gly Trp Gly Asn Gly Cys Gly Phe Phe Gly Lys Gly Ser	
385 390 395 400	
att gac aca tgt gca aaa ttc tcc tgc acc agt aaa gcg att ggg aga	1366
Ile Asp Thr Cys Ala Lys Phe Ser Cys Thr Ser Lys Ala Ile Gly Arg	
405 410 415	
aca atc cag cca gaa aac atc aaa tac aaa gtt ggc att ttt gtg cat	1414
Thr Ile Gln Pro Glu Asn Ile Lys Tyr Lys Val Gly Ile Phe Val His	
420 425 430	
gga acc acc act tcg gaa aac cat ggg aat tat tca gcg caa gtt ggg	1462
Gly Thr Thr Thr Ser Glu Asn His Gly Asn Tyr Ser Ala Gln Val Gly	
435 440 445	
gcg tcc cag gcg gca aag ttt aca gta aca ccc aat gct cct tcg gta	1510
Ala Ser Gln Ala Ala Lys Phe Thr Val Thr Pro Asn Ala Pro Ser Val	
450 455 460	
gcc ctc aaa ctt ggt gac tac gga gaa gtc aca ctg gac tgt gag cca	1558
Ala Leu Lys Leu Gly Asp Tyr Gly Glu Val Thr Leu Asp Cys Glu Pro	
465 470 475 480	
agg agt gga ctg aac act gaa gcg ttt tac gtc atg acc gtg ggg tca	1606
Arg Ser Gly Leu Asn Thr Glu Ala Phe Tyr Val Met Thr Val Gly Ser	

gtc ttc aac tcc ata gga aga gcc gtt cac caa gtg ttt ggt ggt gcc	2326
Val Phe Asn Ser Ile Gly Arg Ala Val His Gln Val Phe Gly Gly Ala	
725 730 735	
ttc aga aca ctc ttt ggg gga atg tct tgg atc aca caa ggg cta atg	2374
Phe Arg Thr Leu Phe Gly Gly Met Ser Trp Ile Thr Gln Gly Leu Met	
740 745 750	
ggt gcc cta ctg ctc tgg atg ggc gtc aac gca cga gac cga tca att	2422
Gly Ala Leu Leu Leu Trp Met Gly Val Asn Ala Arg Asp Arg Ser Ile	
755 760 765	
gct ttg gcc ttc tta gcc aca gga ggt gtg ctc gtg ttc tta gcg acc	2470
Ala Leu Ala Phe Leu Ala Thr Gly Gly Val Leu Val Phe Leu Ala Thr	
770 775 780	
aat gtg ggc gcc gat caa gga tgc gcc atc aac ttt ggc aag aga gag	2518
Asn Val Gly Ala Asp Gln Gly Cys Ala Ile Asn Phe Gly Lys Arg Glu	
785 790 795 800	
ctc aag tgc gga gat ggt atc ttc ata ttt aga gac tct gat gac tgg	2566
Leu Lys Cys Gly Asp Gly Ile Phe Ile Phe Arg Asp Ser Asp Asp Trp	
805 810 815	
ctg aac aag tac tca tac tat cca gaa gat cct gtg aag ctt gca tca	2614
Leu Asn Lys Tyr Ser Tyr Tyr Pro Glu Asp Pro Val Lys Leu Ala Ser	
820 825 830	
ata gtg aaa gcc tct ttt gaa gaa ggg aag tgt ggc cta aat tca gtt	2662
Ile Val Lys Ala Ser Phe Glu Glu Gly Lys Cys Gly Leu Asn Ser Val	
835 840 845	
gac tcc ctt gag cat gag atg tgg aga agc agg gca gat gag atc aat	2710
Asp Ser Leu Glu His Glu Met Trp Arg Ser Arg Ala Asp Glu Ile Asn	
850 855 860	
gcc att ttt gag gaa aac gag gtg gac att tct gtt gtc gtg cag gat	2758
Ala Ile Phe Glu Glu Asn Glu Val Asp Ile Ser Val Val Val Gln Asp	
865 870 875 880	
cca aag aat gtt tac cag aga gga act cat cca ttt tcc aga att cgg	2806
Pro Lys Asn Val Tyr Gln Arg Gly Thr His Pro Phe Ser Arg Ile Arg	
885 890 895	
gat ggt ctg cag tat ggt tgg aag act tgg ggt aag aac ctt gtg ttc	2854
Asp Gly Leu Gln Tyr Gly Trp Lys Thr Trp Gly Lys Asn Leu Val Phe	
900 905 910	
tcc cca ggg agg aag aat gga agc ttc atc ata gat gga aag tcc agg	2902
Ser Pro Gly Arg Lys Asn Gly Ser Phe Ile Ile Asp Gly Lys Ser Arg	
915 920 925	
aaa gaa tgc ccg ttt tca aac cgg gtc tgg aat tct ttc cag ata gag	2950
Lys Glu Cys Pro Phe Ser Asn Arg Val Trp Asn Ser Phe Gln Ile Glu	
930 935 940	
gag ttt ggg acg gga gtg ttc acc aca cgc gtg tac atg gac gca gtc	2998
Glu Phe Gly Thr Gly Val Phe Thr Thr Arg Val Tyr Met Asp Ala Val	

945	950	955	960	
ttt gaa tac acc	ata gac tgc gat	gga tct atc ttg	ggt gca gcg gtt	3046
Phe Glu Tyr Thr	Ile Asp Cys Asp	Gly Ser Ile Leu	Gly Ala Ala Val	
	965	970	975	
aac gga aaa aag	agt gcc cat ggc	tct cca aca ttt	tgg atg gga agt	3094
Asn Gly Lys Lys	Ser Ala His Gly	Ser Pro Thr Phe	Trp Met Gly Ser	
	980	985	990	
cat gaa gta aat	ggg aca tgg atg	atc cac acc ttg	gag gca tta gat	3142
His Glu Val Asn	Gly Thr Trp Met	Ile His Thr Leu	Glu Ala Leu Asp	
	995	1000	1005	
tac aag gag tgt	gag tgg cca ctg	aca cat acg att	gga aca tca gtt	3190
Tyr Lys Glu Cys	Glu Trp Pro Leu	Thr His Thr Ile	Gly Thr Ser Val	
	1010	1015	1020	
gaa gag agt gaa	atg ttc atg ccg	aga tca atc gga	ggc cca gtt agc	3238
Glu Glu Ser Glu	Met Phe Met Pro	Arg Ser Ile Gly	Gly Pro Val Ser	
	1025	1030	1035	1040
tct cac aat cat	atc cct gga tac	aag gtt cag acg	aac gga cct tgg	3286
Ser His Asn His	Ile Pro Gly Tyr	Lys Val Gln Thr	Asn Gly Pro Trp	
	1045	1050	1055	
atg cag gta cca	cta gaa gtg aag	aga gaa gct tgc	cca ggg act agc	3334
Met Gln Val Pro	Leu Glu Val Lys	Arg Glu Ala Cys	Pro Gly Thr Ser	
	1060	1065	1070	
gtg atc att gat	ggc aac tgt gat	gga cgg gga aaa	tca acc aga tcc	3382
Val Ile Ile Asp	Gly Asn Cys Asp	Gly Arg Gly Lys	Ser Thr Arg Ser	
	1075	1080	1085	
acc acg gat agc	ggg aaa gtt att	cct gaa tgg tgt	tgc cgc tcc tgc	3430
Thr Thr Asp Ser	Gly Lys Val Ile	Pro Glu Trp Cys	Cys Arg Ser Cys	
	1090	1095	1100	
aca atg ccg cct	gtg agc ttc cat	ggt agt gat ggg	tgt tgg tat ccc	3478
Thr Met Pro Pro	Val Ser Phe His	Gly Ser Asp Gly	Cys Trp Tyr Pro	
	1105	1110	1115	1120
atg gaa att agg	cca agg aaa acg	cat gaa agc cat	ctg gtg cgc tcc	3526
Met Glu Ile Arg	Pro Arg Lys Thr	His Glu Ser His	Leu Val Arg Ser	
	1125	1130	1135	
tgg gtt aca gct	gga gaa ata cat	gct gtc cct ttt	ggt ttg gtg agc	3574
Trp Val Thr Ala	Gly Glu Ile His	Ala Val Pro Phe	Gly Leu Val Ser	
	1140	1145	1150	
atg atg ata gca	atg gaa gtg gtc	cta agg aaa aga	cag gga cca aag	3622
Met Met Ile Ala	Met Glu Val Val	Leu Arg Lys Arg	Gln Gly Pro Lys	
	1155	1160	1165	
caa atg ttg gtt	gga gga gta gtg	ctc ttg gga gca	atg ctg gtc ggg	3670
Gln Met Leu Val	Gly Gly Val Val	Leu Leu Gly Ala	Met Leu Val Gly	
	1170	1175	1180	

caa gta act ctc ctt gat ttg ctg aaa ctc aca gtg gct gtg gga ttg	3718
Gln Val Thr Leu Leu Asp Leu Leu Lys Leu Thr Val Ala Val Gly Leu	
1185 1190 1195 1200	
cat ttc cat gag atg aac aat gga gga gac gcc atg tat atg gcg ttg	3766
His Phe His Glu Met Asn Asn Gly Gly Asp Ala Met Tyr Met Ala Leu	
1205 1210 1215	
att gct gcc ttt tca atc aga cca ggg ctg ctc atc ggc ttt ggg ctc	3814
Ile Ala Ala Phe Ser Ile Arg Pro Gly Leu Leu Ile Gly Phe Gly Leu	
1220 1225 1230	
agg acc cta tgg agc cct cgg gaa cgc ctt gtg ctg acc cta gga gca	3862
Arg Thr Leu Trp Ser Pro Arg Leu Val Leu Thr Leu Gly Ala	
1235 1240 1245	
gcc atg gtg gag att gcc ttg ggt ggc gtg atg ggc ggc ctg tgg aag	3910
Ala Met Val Glu Ile Ala Leu Gly Gly Val Met Gly Gly Leu Trp Lys	
1250 1255 1260	
tat cta aat gca gtt tct ctc tgc atc ctg aca ata aat gct gtt gct	3958
Tyr Leu Asn Ala Val Ser Leu Cys Ile Leu Thr Ile Asn Ala Val Ala	
1265 1270 1275 1280	
tct agg aaa gca tca aat acc atc ttg ccc ctc atg gct ctg ttg aca	4006
Ser Arg Lys Ala Ser Asn Thr Ile Leu Pro Leu Met Ala Leu Leu Thr	
1285 1290 1295	
cct gtc act atg gct gag gtg aga ctt gcc gca atg ttc ttt tgt gcc	4054
Pro Val Thr Met Ala Glu Val Arg Leu Ala Ala Met Phe Phe Cys Ala	
1300 1305 1310	
atg gtt atc ata ggg gtc ctt cac cag aat ttc aag gac acc tcc atg	4102
Met Val Ile Ile Gly Val Leu His Gln Asn Phe Lys Asp Thr Ser Met	
1315 1320 1325	
cag aag act ata cct ctg gtg gcc ctc aca ctc aca tct tac ctg ggc	4150
Gln Lys Thr Ile Pro Leu Val Ala Leu Thr Leu Thr Ser Tyr Leu Gly	
1330 1335 1340	
ttg aca caa cct ttt ttg ggc ctg tgt gca ttt ctg gca acc cgc ata	4198
Leu Thr Gln Pro Phe Leu Gly Leu Cys Ala Phe Leu Ala Thr Arg Ile	
1345 1350 1355 1360	
ttt ggg cga agg agt atc cca gtg aat gag gca ctc gca gca gct ggt	4246
Phe Gly Arg Arg Ser Ile Pro Val Asn Glu Ala Leu Ala Ala Gly	
1365 1370 1375	
cta gtg gga gtg ctg gca gga ctg gct ttt cag gag atg gag aac ttc	4294
Leu Val Gly Val Leu Ala Gly Leu Ala Phe Gln Glu Met Glu Asn Phe	
1380 1385 1390	
ctt ggt ccg att gca gtt gga gga ctc ctg atg atg ctg gtt agc gtg	4342
Leu Gly Pro Ile Ala Val Gly Gly Leu Leu Met Met Leu Val Ser Val	
1395 1400 1405	
gct ggg agg gtg gat ggg cta gag ctc aag aag ctt ggt gaa gtt tca	4390
Ala Gly Arg Val Asp Gly Leu Glu Leu Lys Lys Leu Gly Glu Val Ser	

1410	1415	1420	
tgg gaa gag gag gcg gag atc agc ggg agt tcc gcc cgc tat gat gtg			4438
Trp Glu Glu Glu Ala Glu Ile Ser Gly Ser Ser Ala Arg Tyr Asp Val			
1425	1430	1435	1440
gca ctc agt gaa caa ggg gag ttc aag ctg ctt tct gaa gag aaa gtg			4486
Ala Leu Ser Glu Gln Gly Glu Phe Lys Leu Leu Ser Glu Glu Lys Val			
	1445	1450	1455
cca tgg gac cag gtt gtg atg acc tcg ctg gcc ttg gtt ggg gct gcc			4534
Pro Trp Asp Gln Val Val Met Thr Ser Leu Ala Leu Val Gly Ala Ala			
	1460	1465	1470
ctc cat cca ttt gct ctt ctg ctg gtc ctt gct ggg tgg ctg ttt cat			4582
Leu His Pro Phe Ala Leu Leu Leu Val Leu Ala Gly Trp Leu Phe His			
	1475	1480	1485
gtc agg gga gct agg aga agt ggg gat gtc ttg tgg gat att ccc act			4630
Val Arg Gly Ala Arg Arg Ser Gly Asp Val Leu Trp Asp Ile Pro Thr			
	1490	1495	1500
cct aag atc atc gag gaa tgt gaa cat ctg gag gat ggg att tat ggc			4678
Pro Lys Ile Ile Glu Glu Cys Glu His Leu Glu Asp Gly Ile Tyr Gly			
	1505	1510	1520
ata ttc cag tca acc ttc ttg ggg gcc tcc cag cga gga gtg gga gtg			4726
Ile Phe Gln Ser Thr Phe Leu Gly Ala Ser Gln Arg Gly Val Gly Val			
	1525	1530	1535
gca cag gga ggg gtg ttc cac aca atg tgg cat gtc aca aga gga gct			4774
Ala Gln Gly Gly Val Phe His Thr Met Trp His Val Thr Arg Gly Ala			
	1540	1545	1550
ttc ctt gtc agg aat ggc aag aag ttg att cca tct tgg gct tca gta			4822
Phe Leu Val Arg Asn Gly Lys Lys Leu Ile Pro Ser Trp Ala Ser Val			
	1555	1560	1565
aag gaa gac ctt gtc gcc tat ggt ggc tca tgg aag ttg gaa ggc aga			4870
Lys Glu Asp Leu Val Ala Tyr Gly Gly Ser Trp Lys Leu Glu Gly Arg			
	1570	1575	1580
tgg gat gga gag gaa gag gtc cag ttg atc gcg gct gtt cca gga aag			4918
Trp Asp Gly Glu Glu Glu Val Gln Leu Ile Ala Ala Val Pro Gly Lys			
	1585	1590	1600
aac gtg gtc aac gtc cag aca aaa ccg agc ttg ttc aaa gtg agg aat			4966
Asn Val Val Asn Val Gln Thr Lys Pro Ser Leu Phe Lys Val Arg Asn			
	1605	1610	1615
ggg gga gaa atc ggg gct gtc gct ctt gac tat ccg agt ggc act tca			5014
Gly Gly Glu Ile Gly Ala Val Ala Leu Asp Tyr Pro Ser Gly Thr Ser			
	1620	1625	1630
gga tct cct att gtt aac agg aac gga gag gtg att ggg ctg tac ggc			5062
Gly Ser Pro Ile Val Asn Arg Asn Gly Glu Val Ile Gly Leu Tyr Gly			
	1635	1640	1645

aat ggc atc ctt gtc ggt gac aac tcc ttc gtg tcc gcc ata tcc cag	5110
Asn Gly Ile Leu Val Gly Asp Asn Ser Phe Val Ser Ala Ile Ser Gln	
1650 1655 1660	
act gag gtg aag gaa gaa gga aag gag gag ctc caa gag atc ccg aca	5158
Thr Glu Val Lys Glu Glu Gly Lys Glu Glu Leu Gln Glu Ile Pro Thr	
1665 1670 1675 1680	
atg cta aag aaa gga atg aca act gtc ctt gat ttt cat cct gga gct	5206
Met Leu Lys Lys Gly Met Thr Thr Val Leu Asp Phe His Pro Gly Ala	
1685 1690 1695	
ggg aag aca aga cgt ttc ctc cca cag atc ttg gcc gag tgc gca cgg	5254
Gly Lys Thr Arg Arg Phe Leu Pro Gln Ile Leu Ala Glu Cys Ala Arg	
1700 1705 1710	
aga cgc ttg cgc act ctt gtg ttg gcc ccc acc agg gtt gtt ctt tct	5302
Arg Arg Leu Arg Thr Leu Val Leu Ala Pro Thr Arg Val Val Leu Ser	
1715 1720 1725	
gaa atg aag gag gct ttt cac ggc ctg gac gtg aaa ttc cac aca cag	5350
Glu Met Lys Glu Ala Phe His Gly Leu Asp Val Lys Phe His Thr Gln	
1730 1735 1740	
gct ttt tcc gct cac ggc agc ggg aga gaa gtc att gat gcc atg tgc	5398
Ala Phe Ser Ala His Gly Ser Gly Arg Glu Val Ile Asp Ala Met Cys	
1745 1750 1755 1760	
cat gcc acc cta act tac agg atg ttg gaa cca act agg gtt gtt aac	5446
His Ala Thr Leu Thr Tyr Arg Met Leu Glu Pro Thr Arg Val Val Asn	
1765 1770 1775	
tgg gaa gtg atc att atg gat gaa gcc cat ttt ttg gat cca gct agc	5494
Trp Glu Val Ile Ile Met Asp Glu Ala His Phe Leu Asp Pro Ala Ser	
1780 1785 1790	
ata gcc gct aga ggt tgg gca gcg cac aga gct agg gca aat gaa agt	5542
Ile Ala Ala Arg Gly Trp Ala Ala His Arg Ala Arg Ala Asn Glu Ser	
1795 1800 1805	
gca aca atc ttg atg aca gcc aca ccg cct ggg act agt gat gaa ttt	5590
Ala Thr Ile Leu Met Thr Ala Thr Pro Pro Gly Thr Ser Asp Glu Phe	
1810 1815 1820	
cca cat tca aat ggt gaa ata gaa gat gtt caa acg gac ata ccc agt	5638
Pro His Ser Asn Gly Glu Ile Glu Asp Val Gln Thr Asp Ile Pro Ser	
1825 1830 1835 1840	
gag ccc tgg aac aca ggg cat gac tgg atc ctg gct gac aaa agg ccc	5686
Glu Pro Trp Asn Thr Gly His Asp Trp Ile Leu Ala Asp Lys Arg Pro	
1845 1850 1855	
acg gca tgg ttc ctt cca tcc atc aga gct gca aat gtc atg gct gcc	5734
Thr Ala Trp Phe Leu Pro Ser Ile Arg Ala Ala Asn Val Met Ala Ala	
1860 1865 1870	
tct ttg cgt aag gct gga aag agt gtg gtg gtc ctg aac agg aaa acc	5782
Ser Leu Arg Lys Ala Gly Lys Ser Val Val Val Leu Asn Arg Lys Thr	

1875	1880	1885	
ttt gag aga gaa tac ccc acg ata aag cag aag aaa cct gac ttt ata Phe Glu Arg Glu Tyr Pro Thr Ile Lys Gln Lys Lys Pro Asp Phe Ile 1890 1895 1900			5830
ttg gcc act gac ata gct gaa atg gga gcc aac ctt tgc gtg gag cga Leu Ala Thr Asp Ile Ala Glu Met Gly Ala Asn Leu Cys Val Glu Arg 1905 1910 1915 1920			5878
gtg ctg gat tgc agg acg gct ttt aag cct gtg ctt gtg gat gaa ggg Val Leu Asp Cys Arg Thr Ala Phe Lys Pro Val Leu Val Asp Glu Gly 1925 1930 1935			5926
agg aag gtg gca ata aaa ggg cca ctt cgt atc tcc gca tcc tct gct Arg Lys Val Ala Ile Lys Gly Pro Leu Arg Ile Ser Ala Ser Ser Ala 1940 1945 1950			5974
gct caa agg agg ggg cgc att ggg aga aat ccc aac aga gat gga gac Ala Gln Arg Arg Gly Arg Ile Gly Arg Asn Pro Asn Arg Asp Gly Asp 1955 1960 1965			6022
tca tac tac tat tct gag cct aca agt gaa aat aat gcc cac cac gtc Ser Tyr Tyr Tyr Ser Glu Pro Thr Ser Glu Asn Asn Ala His His Val 1970 1975 1980			6070
tgc tgg ttg gag gcc tca atg ctc ttg gac aac atg gag gtg agg ggt Cys Trp Leu Glu Ala Ser Met Leu Leu Asp Asn Met Glu Val Arg Gly 1985 1990 1995 2000			6118
gga atg gtc gcc cca ctc tat ggc gtt gaa gga act aaa aca cca gtt Gly Met Val Ala Pro Leu Tyr Gly Val Glu Gly Thr Lys Thr Pro Val 2005 2010 2015			6166
tcc cct ggt gaa atg aga ctg agg gat gac cag agg aaa gtc ttc aga Ser Pro Gly Glu Met Arg Leu Arg Asp Asp Gln Arg Lys Val Phe Arg 2020 2025 2030			6214
gaa cta gtg agg aat tgt gac ctg ccc gtt tgg ctt tcg tgg caa gtg Glu Leu Val Arg Asn Cys Asp Leu Pro Val Trp Leu Ser Trp Gln Val 2035 2040 2045			6262
gcc aag gct ggt ttg aag acg aat gat cgt aag tgg tgt ttt gaa ggc Ala Lys Ala Gly Leu Lys Thr Asn Asp Arg Lys Trp Cys Phe Glu Gly 2050 2055 2060			6310
cct gag gaa cat gag atc ttg aat gac agc ggt gaa aca gtg aag tgc Pro Glu Glu His Glu Ile Leu Asn Asp Ser Gly Glu Thr Val Lys Cys 2065 2070 2075 2080			6358
agg gct cct gga gga gca aag aag cct ctg cgc cca agg tgg tgt gat Arg Ala Pro Gly Gly Ala Lys Lys Pro Leu Arg Pro Arg Trp Cys Asp 2085 2090 2095			6406
gaa agg gtg tca tct gac cag agt gcg ctg tct gaa ttt att aag ttt Glu Arg Val Ser Ser Asp Gln Ser Ala Leu Ser Glu Phe Ile Lys Phe 2100 2105 2110			6454

gct gaa ggt agg agg gga gct gct gaa gtg cta gtt gtg ctg agt gaa 6502
 Ala Glu Gly Arg Arg Gly Ala Ala Glu Val Leu Val Val Leu Ser Glu
 2115 2120 2125

ctc cct gat ttc ctg gct aaa aaa ggt gga gag gca atg gat acc atc 6550
 Leu Pro Asp Phe Leu Ala Lys Lys Gly Gly Glu Ala Met Asp Thr Ile
 2130 2135 2140

agt gtg ttc ctc cac tct gag gaa ggc tct agg gct tac cgc aat gca 6598
 Ser Val Phe Leu His Ser Glu Glu Gly Ser Arg Ala Tyr Arg Asn Ala
 2145 2150 2155 2160

cta tca atg atg cct gag gca atg aca ata gtc atg ctg ttt ata ctg 6646
 Leu Ser Met Met Pro Glu Ala Met Thr Ile Val Met Leu Phe Ile Leu
 2165 2170 2175

gct gga cta ctg aca tcg gga atg gtc atc ttt ttc atg tct ccc aaa 6694
 Ala Gly Leu Leu Thr Ser Gly Met Val Ile Phe Phe Met Ser Pro Lys
 2180 2185 2190

ggc atc agt aga atg tct atg gcg atg ggc aca atg gcc ggc tgt gga 6742
 Gly Ile Ser Arg Met Ser Met Ala Met Gly Thr Met Ala Gly Cys Gly
 2195 2200 2205

tat ctc atg ttc ctt gga ggc gtc aaa ccc act cac atc tcc tat gtc 6790
 Tyr Leu Met Phe Leu Gly Gly Val Lys Pro Thr His Ile Ser Tyr Val
 2210 2215 2220

atg ctc ata ttc ttt gtc ctg atg gtg gtt gtg atc ccc gag cca ggg 6838
 Met Leu Ile Phe Phe Val Leu Met Val Val Val Ile Pro Glu Pro Gly
 2225 2230 2235 2240

caa caa agg tcc atc caa gac aac caa gtg gca tac ctc att att ggc 6886
 Gln Gln Arg Ser Ile Gln Asp Asn Gln Val Ala Tyr Leu Ile Ile Gly
 2245 2250 2255

atc ctg acg ctg gtt tca gcg gtg gca gcc aac gag cta ggc atg ctg 6934
 Ile Leu Thr Leu Val Ser Ala Val Ala Ala Asn Glu Leu Gly Met Leu
 2260 2265 2270

gag aaa acc aaa gag gac ctc ttt ggg aag aag aac tta att cca tct 6982
 Glu Lys Thr Lys Glu Asp Leu Phe Gly Lys Lys Asn Leu Ile Pro Ser
 2275 2280 2285

agt gct tca ccc tgg agt tgg ccg gat ctt gac ctg aag cca gga gct 7030
 Ser Ala Ser Pro Trp Ser Trp Pro Asp Leu Asp Leu Lys Pro Gly Ala
 2290 2295 2300

gcc tgg aca gtg tac gtt ggc att gtt aca atg ctc tct cca atg ttg 7078
 Ala Trp Thr Val Tyr Val Gly Ile Val Thr Met Leu Ser Pro Met Leu
 2305 2310 2315 2320

cac cac tgg atc aaa gtc gaa tat ggc aac ctg tct ctg tct gga ata 7126
 His His Trp Ile Lys Val Glu Tyr Gly Asn Leu Ser Leu Ser Gly Ile
 2325 2330 2335

gcc cag tca gcc tca gtc ctt tct ttc atg gac aag ggg ata cca ttc 7174
 Ala Gln Ser Ala Ser Val Leu Ser Phe Met Asp Lys Gly Ile Pro Phe

2340	2345	2350	
atg aag atg aat atc tcg gtc	ata atg ctg ctg gtc	agt ggc tgg aat	7222
Met Lys Met Asn Ile Ser Val	Ile Met Leu Leu Val	Ser Gly Trp Asn	
2355	2360	2365	
tca ata aca gtg atg cct ctg ctc	tgt ggc ata ggg tgc gcc atg ctc		7270
Ser Ile Thr Val Met Pro Leu Leu	Cys Gly Ile Gly Cys Ala Met Leu		
2370	2375	2380	
cac tgg tct ctc att tta cct gga	atc aaa gcg cag cag tca aag ctt		7318
His Trp Ser Leu Ile Leu Pro Gly	Ile Lys Ala Gln Gln Ser Lys Leu		
2385	2390	2395	2400
gca cag aga agg gtg ttc cat ggc	gtt gcc aag aac cct gtg gtt gat		7366
Ala Gln Arg Arg Val Phe His Gly	Val Ala Lys Asn Pro Val Val Asp		
2405	2410	2415	
ggg aat cca aca gtt gac att gag	gaa gct cct gaa atg cct gcc ctt		7414
Gly Asn Pro Thr Val Asp Ile Glu	Glu Ala Pro Glu Met Pro Ala Leu		
2420	2425	2430	
tat gag aag aaa ctg gct cta tat	ctc ctt ctt gct ctc agc cta gct		7462
Tyr Glu Lys Lys Leu Ala Leu Tyr	Leu Leu Leu Ala Leu Ser Leu Ala		
2435	2440	2445	
tct gtt gcc atg tgc aga acg ccc	ttt tca ttg gct gaa ggc att gtc		7510
Ser Val Ala Met Cys Arg Thr Pro	Phe Ser Leu Ala Glu Gly Ile Val		
2450	2455	2460	
cta gca tca gct gcc tta ggg ccg	ctc ata gag gga aac acc agc ctt		7558
Leu Ala Ser Ala Ala Leu Gly Pro	Leu Ile Glu Gly Asn Thr Ser Leu		
2465	2470	2475	2480
ctt tgg aat gga ccc atg gct gtc	tcc atg aca gga gtc atg agg ggg		7606
Leu Trp Asn Gly Pro Met Ala Val	Ser Met Thr Gly Val Met Arg Gly		
2485	2490	2495	
aat cac tat gct ttt gtg gga gtc	atg tac aat cta tgg aag atg aaa		7654
Asn His Tyr Ala Phe Val Gly Val	Met Tyr Asn Leu Trp Lys Met Lys		
2500	2505	2510	
act gga cgc cgg ggg agc gcg aat	gga aaa act ttg ggt gaa gtc tgg		7702
Thr Gly Arg Arg Gly Ser Ala Asn	Gly Lys Thr Leu Gly Glu Val Trp		
2515	2520	2525	
aag agg gaa ctg aat ctg ttg gac	aag cga cag ttt gag ttg tat aaa		7750
Lys Arg Glu Leu Asn Leu Leu Asp	Lys Arg Gln Phe Glu Leu Tyr Lys		
2530	2535	2540	
agg acc gac att gtg gag gtg gat	cgt gat acg gca cgc agg cat ttg		7798
Arg Thr Asp Ile Val Glu Val Asp	Arg Asp Thr Ala Arg Arg His Leu		
2545	2550	2555	2560
gcc gaa ggg aag gtg gac acc ggg	gtg gcg gtc tcc agg ggg acc gca		7846
Ala Glu Gly Lys Val Asp Thr Gly	Val Ala Val Ser Arg Gly Thr Ala		
2565	2570	2575	

aag tta agg tgg ttc cat gag cgt ggc tat gtc aag ctg gaa ggt agg	7894
Lys Leu Arg Trp Phe His Glu Arg Gly Tyr Val Lys Leu Glu Gly Arg	
2580 2585 2590	
gtg att gac ctg ggg tgt ggc cgc gga ggc tgg tgt tac tac gct gct	7942
Val Ile Asp Leu Gly Cys Gly Arg Gly Gly Trp Cys Tyr Tyr Ala Ala	
2595 2600 2605	
gcg caa aag gaa gtg agt ggg gtc aaa gga ttt act ctt gga aga gac	7990
Ala Gln Lys Glu Val Ser Gly Val Lys Gly Phe Thr Leu Gly Arg Asp	
2610 2615 2620	
ggc cat gag aaa ccc atg aat gtg caa agt ctg gga tgg aac atc atc	8038
Gly His Glu Lys Pro Met Asn Val Gln Ser Leu Gly Trp Asn Ile Ile	
2625 2630 2635 2640	
acc ttc aag gac aaa act gat atc cac cgc cta gaa cca gtg aaa tgt	8086
Thr Phe Lys Asp Lys Thr Asp Ile His Arg Leu Glu Pro Val Lys Cys	
2645 2650 2655	
gac acc ctt ttg tgt gac att gga gag tca tca tcg tca tcg gtc aca	8134
Asp Thr Leu Leu Cys Asp Ile Gly Glu Ser Ser Ser Ser Ser Val Thr	
2660 2665 2670	
gag ggg gaa agg acc gtg aga gtt ctt gat act gta gaa aaa tgg ctg	8182
Glu Gly Glu Arg Thr Val Arg Val Leu Asp Thr Val Glu Lys Trp Leu	
2675 2680 2685	
gct tgt ggg gtt gac aac ttc tgt gtg aag gtg tta gct cca tac atg	8230
Ala Cys Gly Val Asp Asn Phe Cys Val Lys Val Leu Ala Pro Tyr Met	
2690 2695 2700	
cca gat gtt ctt gag aaa ctg gaa ttg ctc caa agg agg ttt ggc gga	8278
Pro Asp Val Leu Glu Lys Leu Glu Leu Leu Gln Arg Arg Phe Gly Gly	
2705 2710 2715 2720	
aca gtg atc agg aac cct ctc tcc agg aat tcc act cat gaa atg tac	8326
Thr Val Ile Arg Asn Pro Leu Ser Arg Asn Ser Thr His Glu Met Tyr	
2725 2730 2735	
tac gtg tct gga gcc cgc agc aat gtc aca ttt act gtg aac caa aca	8374
Tyr Val Ser Gly Ala Arg Ser Asn Val Thr Phe Thr Val Asn Gln Thr	
2740 2745 2750	
tcc cgc ctc ctg atg agg aga atg agg cgt cca act gga aaa gtg acc	8422
Ser Arg Leu Leu Met Arg Arg Met Arg Arg Pro Thr Gly Lys Val Thr	
2755 2760 2765	
ctg gag gct gac gtc atc ctc cca att ggg aca cgc agt gtt gag aca	8470
Leu Glu Ala Asp Val Ile Leu Pro Ile Gly Thr Arg Ser Val Glu Thr	
2770 2775 2780	
gac aag gga ccc ctg gac aaa gag gcc ata gaa gaa agg gtt gag agg	8518
Asp Lys Gly Pro Leu Asp Lys Glu Ala Ile Glu Glu Arg Val Glu Arg	
2785 2790 2795 2800	
ata aaa tct gag tac atg acc tct tgg ttt tat gac aat gac aac ccc	8566
Ile Lys Ser Glu Tyr Met Thr Ser Trp Phe Tyr Asp Asn Asp Asn Pro	

2805										2810					2815					
tac	agg	acc	tgg	cac	tac	tgt	ggc	tcc	tat	gtc	aca	aaa	acc	tcc	gga	8614				
Tyr	Arg	Thr	Trp	His	Tyr	Cys	Gly	Ser	Tyr	Val	Thr	Lys	Thr	Ser	Gly					
			2820					2825					2830							
agt	gcg	gcg	agc	atg	gta	aat	ggt	gtt	att	aaa	att	ctg	aca	tat	cca	8662				
Ser	Ala	Ala	Ser	Met	Val	Asn	Gly	Val	Ile	Lys	Ile	Leu	Thr	Tyr	Pro					
			2835				2840					2845								
tgg	gac	agg	ata	gag	gag	gtc	aca	aga	atg	gca	atg	act	gac	aca	acc	8710				
Trp	Asp	Arg	Ile	Glu	Glu	Val	Thr	Arg	Met	Ala	Met	Thr	Asp	Thr	Thr					
	2850					2855				2860										
cct	ttt	gga	cag	caa	aga	gtg	ttt	aaa	gaa	aaa	gtt	gac	acc	aga	gca	8758				
Pro	Phe	Gly	Gln	Gln	Arg	Val	Phe	Lys	Glu	Lys	Val	Asp	Thr	Arg	Ala					
2865					2870				2875						2880					
aag	gat	cca	cca	gcg	gga	act	agg	aag	atc	atg	aaa	gtt	gtc	aac	agg	8806				
Lys	Asp	Pro	Pro	Ala	Gly	Thr	Arg	Lys	Ile	Met	Lys	Val	Val	Asn	Arg					
				2885				2890						2895						
tgg	ctg	ttc	cgc	cac	ctg	gcc	aga	gaa	aag	aac	ccc	aga	ctg	tgc	aca	8854				
Trp	Leu	Phe	Arg	His	Leu	Ala	Arg	Glu	Lys	Asn	Pro	Arg	Leu	Cys	Thr					
			2900					2905					2910							
aag	gaa	gaa	ttt	att	gca	aaa	gtc	cga	agt	cat	gca	gcc	att	gga	gct	8902				
Lys	Glu	Glu	Phe	Ile	Ala	Lys	Val	Arg	Ser	His	Ala	Ala	Ile	Gly	Ala					
	2915					2920					2925									
tac	ctg	gaa	gaa	caa	gaa	cag	tgg	aag	act	gcc	aat	gag	gct	gtc	caa	8950				
Tyr	Leu	Glu	Glu	Gln	Glu	Gln	Trp	Lys	Thr	Ala	Asn	Glu	Ala	Val	Gln					
	2930					2935				2940										
gac	cca	aag	ttc	tgg	gaa	ctg	gtg	gat	gaa	gaa	agg	aag	ctg	cac	caa	8998				
Asp	Pro	Lys	Phe	Trp	Glu	Leu	Val	Asp	Glu	Glu	Arg	Lys	Leu	His	Gln					
2945					2950				2955					2960						
caa	ggc	agg	tgt	cgg	act	tgt	gtg	tac	aac	atg	atg	ggg	aaa	aga	gag	9046				
Gln	Gly	Arg	Cys	Arg	Thr	Cys	Val	Tyr	Asn	Met	Met	Gly	Lys	Arg	Glu					
				2965				2970					2975							
aag	aag	ctg	tca	gag	ttt	ggg	aaa	gca	aag	gga	agc	cgt	gcc	ata	tgg	9094				
Lys	Lys	Leu	Ser	Glu	Phe	Gly	Lys	Ala	Lys	Gly	Ser	Arg	Ala	Ile	Trp					
			2980					2985					2990							
tat	atg	tgg	ctg	gga	gcg	cgg	tat	ctt	gag	ttt	gag	gcc	ctg	gga	ttc	9142				
Tyr	Met	Trp	Leu	Gly	Ala	Arg	Tyr	Leu	Glu	Phe	Glu	Ala	Leu	Gly	Phe					
	2995						3000					3005								
ctg	aat	gag	gac	cat	tgg	gct	tcc	agg	gaa	aac	tca	gga	gga	gga	gtg	9190				
Leu	Asn	Glu	Asp	His	Trp	Ala	Ser	Arg	Glu	Asn	Ser	Gly	Gly	Gly	Val					
	3010					3015					3020									
gaa	ggc	att	ggc	tta	caa	tac	cta	gga	tat	gtg	atc	aga	gac	ctg	gct	9238				
Glu	Gly	Ile	Gly	Leu	Gln	Tyr	Leu	Gly	Tyr	Val	Ile	Arg	Asp	Leu	Ala					
3025				3030					3035					3040						

gca atg gat ggt ggt gga ttc tac gcg gat gac acc gct gga tgg gac 9286
 Ala Met Asp Gly Gly Gly Phe Tyr Ala Asp Asp Thr Ala Gly Trp Asp
 3045 3050 3055

acg cgc atc aca gag gca gac ctt gat gat gaa cag gag atc ttg aac 9334
 Thr Arg Ile Thr Glu Ala Asp Leu Asp Asp Glu Gln Glu Ile Leu Asn
 3060 3065 3070

tac atg agc cca cat cac aaa aaa ctg gca caa gca gtg atg gaa atg 9382
 Tyr Met Ser Pro His His Lys Lys Leu Ala Gln Ala Val Met Glu Met
 3075 3080 3085

aca tac aag aac aaa gtg gtg aaa gtg ttg aga cca gcc cca gga ggg 9430
 Thr Tyr Lys Asn Lys Val Val Lys Val Leu Arg Pro Ala Pro Gly Gly
 3090 3095 3100

aaa gcc tac atg gat gtc ata agt cga cga gac cag aga gga tcc ggg 9478
 Lys Ala Tyr Met Asp Val Ile Ser Arg Arg Asp Gln Arg Gly Ser Gly
 3105 3110 3115 3120

cag gta gtg act tat gct ctg aac acc atc acc aac ttg aaa gtc caa 9526
 Gln Val Val Thr Tyr Ala Leu Asn Thr Ile Thr Asn Leu Lys Val Gln
 3125 3130 3135

ttg atc aga atg gca gaa gca gag atg gtg ata cat cac caa cat gtt 9574
 Leu Ile Arg Met Ala Glu Ala Glu Met Val Ile His His Gln His Val
 3140 3145 3150

caa gat tgt gat gaa tca gtt ctg acc agg ctg gag gca tgg ctc act 9622
 Gln Asp Cys Asp Glu Ser Val Leu Thr Arg Leu Glu Ala Trp Leu Thr
 3155 3160 3165

gag cac gga tgt gac aga ctg aag agg atg gcg gtg agt gga gac gac 9670
 Glu His Gly Cys Asp Arg Leu Lys Arg Met Ala Val Ser Gly Asp Asp
 3170 3175 3180

tgt gtg gtc cgg ccc atc gat gac agg ttc ggc ctg gcc ctg tcc cat 9718
 Cys Val Val Arg Pro Ile Asp Asp Arg Phe Gly Leu Ala Leu Ser His
 3185 3190 3195 3200

ctc aac gcc atg tcc aag gtt aga aag gac ata tct gaa tgg cag cca 9766
 Leu Asn Ala Met Ser Lys Val Arg Lys Asp Ile Ser Glu Trp Gln Pro
 3205 3210 3215

tca aaa ggg tgg aat gat tgg gag aat gtg ccc ttc tgt tcc cac cac 9814
 Ser Lys Gly Trp Asn Asp Trp Glu Asn Val Pro Phe Cys Ser His His
 3220 3225 3230

ttc cat gaa cta cag ctg aag gat ggc agg agg att gtg gtg cct tgc 9862
 Phe His Glu Leu Gln Leu Lys Asp Gly Arg Arg Ile Val Val Pro Cys
 3235 3240 3245

cga gaa cag gac gag ctc att ggg aga gga agg gtg tct cca gga aac 9910
 Arg Glu Gln Asp Glu Leu Ile Gly Arg Gly Arg Val Ser Pro Gly Asn
 3250 3255 3260

ggc tgg atg atc aag gaa aca gct tgc ctc agc aaa gcc tat gcc aac 9958
 Gly Trp Met Ile Lys Glu Thr Ala Cys Leu Ser Lys Ala Tyr Ala Asn

3265	3270	3275	3280	
atg tgg tca ctg atg tat ttt cac aaa agg gac atg agg cta ctg tca				10006
Met Trp Ser Leu Met Tyr Phe His Lys Arg Asp Met Arg Leu Leu Ser	3285	3290	3295	
ttg gct gtt tcc tca gct gtt ccc acc tca tgg gtt cca caa gga cgc				10054
Leu Ala Val Ser Ser Ala Val Pro Thr Ser Trp Val Pro Gln Gly Arg	3300	3305	3310	
aca aca tgg tgc att cat ggg aaa ggg gag tgg atg acc acg gaa gac				10102
Thr Thr Trp Ser Ile His Gly Lys Gly Glu Trp Met Thr Thr Glu Asp	3315	3320	3325	
atg ctt gag gtg tgg aac aga gta tgg ata acc aac aac cca cac atg				10150
Met Leu Glu Val Trp Asn Arg Val Trp Ile Thr Asn Asn Pro His Met	3330	3335	3340	
cag gac aag aca atg gtg aaa aaa tgg aga gat gtc cct tat cta acc				10198
Gln Asp Lys Thr Met Val Lys Lys Trp Arg Asp Val Pro Tyr Leu Thr	3345	3350	3355	3360
aag aga caa gac aag ctg tgc gga tca ctg att gga atg acc aat agg				10246
Lys Arg Gln Asp Lys Leu Cys Gly Ser Leu Ile Gly Met Thr Asn Arg	3365	3370	3375	
gcc acc tgg gcc tcc cac atc cat tta gtc atc cat cgt atc cga acg				10294
Ala Thr Trp Ala Ser His Ile His Leu Val Ile His Arg Ile Arg Thr	3380	3385	3390	
ctg att gga cag gag aaa tac act gac tac cta aca gtc atg gac agg				10342
Leu Ile Gly Gln Glu Lys Tyr Thr Asp Tyr Leu Thr Val Met Asp Arg	3395	3400	3405	
tat tct gtg gat gct gac ctg caa ctg ggt gag ctt atc tgaaacacca				10391
Tyr Ser Val Asp Ala Asp Leu Gln Leu Gly Glu Leu Ile	3410	3415	3420	
tctaacagga ataaccggga tacaaccac ggggtggagaa ccggactccc cacaacctga				10451
aaccgggata taaaccacgg ctggagaacc gggctccgca cttaaaatga aacagaaacc				10511
gggataaaaa ctacggatgg agaaccggac tccacacatt gagacagaag aagttgtcag				10571
cccagaaccc cacacgagtt ttgccactgc taagctgtga ggcagtgacg gctgggacag				10631
ccgacctcca ggttgcgaaa aacctggttt ctgggacctc ccaccccaga gtaaaaagaa				10691
cggagcctcc gctaccaccc tcccacgtgg tggtagaaag acgggggtcta gaggttagag				10751
gagacctcc agggacaata tagtgggacc atattgacgc caggggaaaga ccggagtggg				10811
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gatgacaaac acaaaaccac t				10892

<210> 53

<211> 3421

<212> PRT

<213> Artificial Sequence

<220>

<223> derived from Yellow Fever virus and Japanese Encephalitis virus

<400> 53

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Arg	Arg	Gly	Val	Arg	Ser	Leu	Ser	Asn	Lys	Ile	Lys	Gln	Lys	Thr	Lys
			20					25					30		
Gln	Ile	Gly	Asn	Arg	Pro	Gly	Pro	Ser	Arg	Gly	Val	Gln	Gly	Phe	Ile
	35					40						45			
Phe	Phe	Phe	Leu	Phe	Asn	Ile	Leu	Thr	Gly	Lys	Lys	Ile	Thr	Ala	His
50					55						60				
Leu	Lys	Arg	Leu	Trp	Lys	Met	Leu	Asp	Pro	Arg	Gln	Gly	Leu	Ala	Val
65					70					75					80
Leu	Arg	Lys	Val	Lys	Arg	Val	Val	Ala	Ser	Leu	Met	Arg	Gly	Leu	Ser
			85						90					95	
Ser	Arg	Lys	Arg	Arg	Ser	His	Asp	Val	Leu	Thr	Val	Gln	Phe	Leu	Ile
			100						105				110		
Leu	Gly	Met	Leu	Leu	Met	Thr	Gly	Gly	Met	Lys	Leu	Ser	Asn	Phe	Gln
		115					120					125			
Gly	Lys	Leu	Leu	Met	Thr	Ile	Asn	Asn	Thr	Asp	Ile	Ala	Asp	Val	Ile
		130				135					140				
Val	Ile	Pro	Thr	Ser	Lys	Gly	Glu	Asn	Arg	Cys	Trp	Val	Arg	Ala	Ile
145					150					155					160
Asp	Val	Gly	Tyr	Met	Cys	Glu	Asp	Thr	Ile	Thr	Tyr	Glu	Cys	Pro	Lys
			165						170					175	
Leu	Thr	Met	Gly	Asn	Asp	Pro	Glu	Asp	Val	Asp	Cys	Trp	Cys	Asp	Asn
			180						185				190		
Gln	Glu	Val	Tyr	Val	Gln	Tyr	Gly	Arg	Cys	Thr	Arg	Thr	Arg	His	Ser
		195					200					205			
Lys	Arg	Ser	Arg	Arg	Ser	Val	Ser	Val	Gln	Thr	His	Gly	Glu	Ser	Ser
		210				215					220				
Leu	Val	Asn	Lys	Lys	Glu	Ala	Trp	Leu	Asp	Ser	Thr	Lys	Ala	Thr	Arg
225					230					235					240
Tyr	Leu	Met	Lys	Thr	Glu	Asn	Trp	Ile	Ile	Arg	Asn	Pro	Gly	Tyr	Ala
			245						250					255	
Phe	Leu	Ala	Ala	Val	Leu	Gly	Trp	Met	Leu	Gly	Ser	Asn	Asn	Gly	Gln
			260					265					270		
Arg	Val	Val	Phe	Thr	Ile	Leu	Leu	Leu	Val	Ala	Pro	Ala	Tyr	Ser	
		275					280					285			
Phe	Asn	Cys	Leu	Gly	Met	Gly	Asn	Arg	Asp	Phe	Ile	Glu	Gly	Ala	Ser
		290				295					300				
Gly	Ala	Thr	Trp	Val	Asp	Leu	Val	Leu	Glu	Gly	Asp	Ser	Cys	Leu	Thr
305					310					315					320
Ile	Met	Ala	Asn	Asp	Lys	Pro	Thr	Leu	Asp	Val	Arg	Met	Ile	Asn	Ile
			325						330					335	
Glu	Ala	Ser	Gln	Leu	Ala	Glu	Val	Arg	Ser	Tyr	Cys	Tyr	His	Ala	Ser
			340					345					350		
Val	Thr	Asp	Ile	Ser	Thr	Val	Ala	Arg	Cys	Pro	Thr	Thr	Gly	Glu	Ala
		355					360					365			
His	Asn	Glu	Lys	Arg	Ala	Asp	Ser	Ser	Tyr	Val	Cys	Lys	Gln	Gly	Phe
		370				375					380				
Thr	Asp	Arg	Gly	Trp	Gly	Asn	Gly	Cys	Gly	Phe	Phe	Gly	Lys	Gly	Ser
385					390					395					400
Ile	Asp	Thr	Cys	Ala	Lys	Phe	Ser	Cys	Thr	Ser	Lys	Ala	Ile	Gly	Arg
			405						410					415	
Thr	Ile	Gln	Pro	Glu	Asn	Ile	Lys	Tyr	Lys	Val	Gly	Ile	Phe	Val	His
			420					425					430		
Gly	Thr	Thr	Thr	Ser	Glu	Asn	His	Gly	Asn	Tyr	Ser	Ala	Gln	Val	Gly
		435					440					445			
Ala	Ser	Gln	Ala	Ala	Lys	Phe	Thr	Val	Thr	Pro	Asn	Ala	Pro	Ser	Val
		450				455					460				

Ala	Leu	Lys	Leu	Gly	Asp	Tyr	Gly	Glu	Val	Thr	Leu	Asp	Cys	Glu	Pro
465					470					475					480
Arg	Ser	Gly	Leu	Asn	Thr	Glu	Ala	Phe	Tyr	Val	Met	Thr	Val	Gly	Ser
				485					490					495	
Lys	Ser	Phe	Leu	Val	His	Arg	Glu	Trp	Phe	His	Asp	Leu	Ala	Leu	Pro
			500					505					510		
Trp	Thr	Ser	Pro	Ser	Ser	Thr	Ala	Trp	Arg	Asn	Arg	Glu	Leu	Leu	Met
		515					520					525			
Glu	Phe	Glu	Gly	Ala	His	Ala	Thr	Lys	Gln	Ser	Val	Val	Ala	Leu	Gly
		530				535					540				
Ser	Gln	Glu	Gly	Gly	Leu	His	His	Ala	Leu	Ala	Gly	Ala	Ile	Val	Val
545					550					555					560
Glu	Tyr	Ser	Ser	Ser	Val	Met	Leu	Thr	Ser	Gly	His	Leu	Lys	Cys	Arg
				565					570					575	
Leu	Lys	Met	Asp	Lys	Leu	Ala	Leu	Lys	Gly	Thr	Thr	Tyr	Gly	Met	Cys
			580					585					590		
Thr	Glu	Lys	Phe	Ser	Phe	Ala	Lys	Asn	Pro	Val	Asp	Thr	Gly	His	Gly
		595					600					605			
Thr	Val	Val	Ile	Glu	Leu	Ser	Tyr	Ser	Gly	Ser	Asp	Gly	Pro	Cys	Lys
		610				615					620				
Ile	Pro	Ile	Val	Ser	Val	Ala	Ser	Leu	Asn	Asp	Met	Thr	Pro	Val	Gly
625					630					635					640
Arg	Leu	Val	Thr	Val	Asn	Pro	Phe	Val	Ala	Thr	Ser	Ser	Ala	Asn	Ser
				645					650					655	
Lys	Val	Leu	Val	Glu	Met	Glu	Pro	Pro	Phe	Gly	Asp	Ser	Tyr	Ile	Val
			660					665					670		
Val	Gly	Arg	Gly	Asp	Lys	Gln	Ile	Asn	His	His	Trp	His	Lys	Ala	Gly
		675				680						685			
Ser	Thr	Leu	Gly	Lys	Ala	Phe	Ser	Thr	Thr	Leu	Lys	Gly	Ala	Gln	Arg
		690				695					700				
Leu	Ala	Ala	Leu	Gly	Asp	Thr	Ala	Trp	Asp	Phe	Gly	Ser	Ile	Gly	Gly
705					710					715					720
Val	Phe	Asn	Ser	Ile	Gly	Arg	Ala	Val	His	Gln	Val	Phe	Gly	Gly	Ala
				725					730					735	
Phe	Arg	Thr	Leu	Phe	Gly	Gly	Met	Ser	Trp	Ile	Thr	Gln	Gly	Leu	Met
			740					745					750		
Gly	Ala	Leu	Leu	Leu	Trp	Met	Gly	Val	Asn	Ala	Arg	Asp	Arg	Ser	Ile
			755				760					765			
Ala	Leu	Ala	Phe	Leu	Ala	Thr	Gly	Gly	Val	Leu	Val	Phe	Leu	Ala	Thr
			770			775					780				
Asn	Val	Gly	Ala	Asp	Gln	Gly	Cys	Ala	Ile	Asn	Phe	Gly	Lys	Arg	Glu
785					790					795					800
Leu	Lys	Cys	Gly	Asp	Gly	Ile	Phe	Ile	Phe	Arg	Asp	Ser	Asp	Asp	Trp
				805					810					815	
Leu	Asn	Lys	Tyr	Ser	Tyr	Tyr	Pro	Glu	Asp	Pro	Val	Lys	Leu	Ala	Ser
			820					825					830		
Ile	Val	Lys	Ala	Ser	Phe	Glu	Glu	Gly	Lys	Cys	Gly	Leu	Asn	Ser	Val
			835				840					845			
Asp	Ser	Leu	Glu	His	Glu	Met	Trp	Arg	Ser	Arg	Ala	Asp	Glu	Ile	Asn
			850			855					860				
Ala	Ile	Phe	Glu	Glu	Asn	Glu	Val	Asp	Ile	Ser	Val	Val	Val	Gln	Asp
865					870					875					880
Pro	Lys	Asn	Val	Tyr	Gln	Arg	Gly	Thr	His	Pro	Phe	Ser	Arg	Ile	Arg
				885					890					895	
Asp	Gly	Leu	Gln	Tyr	Gly	Trp	Lys	Thr	Trp	Gly	Lys	Asn	Leu	Val	Phe
			900					905					910		
Ser	Pro	Gly	Arg	Lys	Asn	Gly	Ser	Phe	Ile	Ile	Asp	Gly	Lys	Ser	Arg
			915				920					925			

Lys Glu Cys Pro Phe Ser Asn Arg Val Trp Asn Ser Phe Gln Ile Glu
 930 935 940
 Glu Phe Gly Thr Gly Val Phe Thr Thr Arg Val Tyr Met Asp Ala Val
 945 950 955 960
 Phe Glu Tyr Thr Ile Asp Cys Asp Gly Ser Ile Leu Gly Ala Ala Val
 965 970 975
 Asn Gly Lys Lys Ser Ala His Gly Ser Pro Thr Phe Trp Met Gly Ser
 980 985 990
 His Glu Val Asn Gly Thr Trp Met Ile His Thr Leu Glu Ala Leu Asp
 995 1000 1005
 Tyr Lys Glu Cys Glu Trp Pro Leu Thr His Thr Ile Gly Thr Ser Val
 1010 1015 1020
 Glu Glu Ser Glu Met Phe Met Pro Arg Ser Ile Gly Gly Pro Val Ser
 1025 1030 1035 1040
 Ser His Asn His Ile Pro Gly Tyr Lys Val Gln Thr Asn Gly Pro Trp
 1045 1050 1055
 Met Gln Val Pro Leu Glu Val Lys Arg Glu Ala Cys Pro Gly Thr Ser
 1060 1065 1070
 Val Ile Ile Asp Gly Asn Cys Asp Gly Arg Gly Lys Ser Thr Arg Ser
 1075 1080 1085
 Thr Thr Asp Ser Gly Lys Val Ile Pro Glu Trp Cys Cys Arg Ser Cys
 1090 1095 1100
 Thr Met Pro Pro Val Ser Phe His Gly Ser Asp Gly Cys Trp Tyr Pro
 1105 1110 1115 1120
 Met Glu Ile Arg Pro Arg Lys Thr His Glu Ser His Leu Val Arg Ser
 1125 1130 1135
 Trp Val Thr Ala Gly Glu Ile His Ala Val Pro Phe Gly Leu Val Ser
 1140 1145 1150
 Met Met Ile Ala Met Glu Val Val Leu Arg Lys Arg Gln Gly Pro Lys
 1155 1160 1165
 Gln Met Leu Val Gly Gly Val Val Leu Leu Gly Ala Met Leu Val Gly
 1170 1175 1180
 Gln Val Thr Leu Leu Asp Leu Leu Lys Leu Thr Val Ala Val Gly Leu
 1185 1190 1195 1200
 His Phe His Glu Met Asn Asn Gly Gly Asp Ala Met Tyr Met Ala Leu
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 Ile Ala Ala Phe Ser Ile Arg Pro Gly Leu Leu Ile Gly Phe Gly Leu
 1220 1225 1230
 Arg Thr Leu Trp Ser Pro Arg Glu Arg Leu Val Leu Thr Leu Gly Ala
 1235 1240 1245
 Ala Met Val Glu Ile Ala Leu Gly Gly Val Met Gly Gly Leu Trp Lys
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 Tyr Leu Asn Ala Val Ser Leu Cys Ile Leu Thr Ile Asn Ala Val Ala
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 Ser Arg Lys Ala Ser Asn Thr Ile Leu Pro Leu Met Ala Leu Leu Thr
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 Pro Val Thr Met Ala Glu Val Arg Leu Ala Ala Met Phe Phe Cys Ala
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 Met Val Ile Ile Gly Val Leu His Gln Asn Phe Lys Asp Thr Ser Met
 1315 1320 1325
 Gln Lys Thr Ile Pro Leu Val Ala Leu Thr Leu Thr Ser Tyr Leu Gly
 1330 1335 1340
 Leu Thr Gln Pro Phe Leu Gly Leu Cys Ala Phe Leu Ala Thr Arg Ile
 1345 1350 1355 1360
 Phe Gly Arg Arg Ser Ile Pro Val Asn Glu Ala Leu Ala Ala Ala Gly
 1365 1370 1375
 Leu Val Gly Val Leu Ala Gly Leu Ala Phe Gln Glu Met Glu Asn Phe
 1380 1385 1390

Leu Gly Pro Ile Ala Val Gly Gly Leu Leu Met Met Leu Val Ser Val
 1395 1400 1405
 Ala Gly Arg Val Asp Gly Leu Glu Leu Lys Lys Leu Gly Glu Val Ser
 1410 1415 1420
 Trp Glu Glu Glu Ala Glu Ile Ser Gly Ser Ser Ala Arg Tyr Asp Val
 1425 1430 1435 1440
 Ala Leu Ser Glu Gln Gly Glu Phe Lys Leu Leu Ser Glu Glu Lys Val
 1445 1450 1455
 Pro Trp Asp Gln Val Val Met Thr Ser Leu Ala Leu Val Gly Ala Ala
 1460 1465 1470
 Leu His Pro Phe Ala Leu Leu Leu Val Leu Ala Gly Trp Leu Phe His
 1475 1480 1485
 Val Arg Gly Ala Arg Arg Ser Gly Asp Val Leu Trp Asp Ile Pro Thr
 1490 1495 1500
 Pro Lys Ile Ile Glu Glu Cys Glu His Leu Glu Asp Gly Ile Tyr Gly
 1505 1510 1515 1520
 Ile Phe Gln Ser Thr Phe Leu Gly Ala Ser Gln Arg Gly Val Gly Val
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 Ala Gln Gly Gly Val Phe His Thr Met Trp His Val Thr Arg Gly Ala
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 Phe Leu Val Arg Asn Gly Lys Lys Leu Ile Pro Ser Trp Ala Ser Val
 1555 1560 1565
 Lys Glu Asp Leu Val Ala Tyr Gly Gly Ser Trp Lys Leu Glu Gly Arg
 1570 1575 1580
 Trp Asp Gly Glu Glu Glu Val Gln Leu Ile Ala Ala Val Pro Gly Lys
 1585 1590 1595 1600
 Asn Val Val Asn Val Gln Thr Lys Pro Ser Leu Phe Lys Val Arg Asn
 1605 1610 1615
 Gly Gly Glu Ile Gly Ala Val Ala Leu Asp Tyr Pro Ser Gly Thr Ser
 1620 1625 1630
 Gly Ser Pro Ile Val Asn Arg Asn Gly Glu Val Ile Gly Leu Tyr Gly
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 Thr Glu Val Lys Glu Gly Lys Glu Glu Leu Gln Glu Ile Pro Thr
 1665 1670 1675 1680
 Met Leu Lys Lys Gly Met Thr Thr Val Leu Asp Phe His Pro Gly Ala
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 Gly Lys Thr Arg Arg Phe Leu Pro Gln Ile Leu Ala Glu Cys Ala Arg
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 Arg Arg Leu Arg Thr Leu Val Leu Ala Pro Thr Arg Val Val Leu Ser
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 Glu Met Lys Glu Ala Phe His Gly Leu Asp Val Lys Phe His Thr Gln
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 Ala Phe Ser Ala His Gly Ser Gly Arg Glu Val Ile Asp Ala Met Cys
 1745 1750 1755 1760
 His Ala Thr Leu Thr Tyr Arg Met Leu Glu Pro Thr Arg Val Val Asn
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 Trp Glu Val Ile Ile Met Asp Glu Ala His Phe Leu Asp Pro Ala Ser
 1780 1785 1790
 Ile Ala Ala Arg Gly Trp Ala Ala His Arg Ala Arg Ala Asn Glu Ser
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 Ala Thr Ile Leu Met Thr Ala Thr Pro Pro Gly Thr Ser Asp Glu Phe
 1810 1815 1820
 Pro His Ser Asn Gly Glu Ile Glu Asp Val Gln Thr Asp Ile Pro Ser
 1825 1830 1835 1840
 Glu Pro Trp Asn Thr Gly His Asp Trp Ile Leu Ala Asp Lys Arg Pro
 1845 1850 1855

Thr Ala Trp Phe Leu Pro Ser Ile Arg Ala Ala Asn Val Met Ala Ala
 1860 1865 1870
 Ser Leu Arg Lys Ala Gly Lys Ser Val Val Val Leu Asn Arg Lys Thr
 1875 1880 1885
 Phe Glu Arg Glu Tyr Pro Thr Ile Lys Gln Lys Lys Pro Asp Phe Ile
 1890 1895 1900
 Leu Ala Thr Asp Ile Ala Glu Met Gly Ala Asn Leu Cys Val Glu Arg
 1905 1910 1915 1920
 Val Leu Asp Cys Arg Thr Ala Phe Lys Pro Val Leu Val Asp Glu Gly
 1925 1930 1935
 Arg Lys Val Ala Ile Lys Gly Pro Leu Arg Ile Ser Ala Ser Ser Ala
 1940 1945 1950
 Ala Gln Arg Arg Gly Arg Ile Gly Arg Asn Pro Asn Arg Asp Gly Asp
 1955 1960 1965
 Ser Tyr Tyr Tyr Ser Glu Pro Thr Ser Glu Asn Asn Ala His His Val
 1970 1975 1980
 Cys Trp Leu Glu Ala Ser Met Leu Leu Asp Asn Met Glu Val Arg Gly
 1985 1990 1995 2000
 Gly Met Val Ala Pro Leu Tyr Gly Val Glu Gly Thr Lys Thr Pro Val
 2005 2010 2015
 Ser Pro Gly Glu Met Arg Leu Arg Asp Asp Gln Arg Lys Val Phe Arg
 2020 2025 2030
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/32821

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 39/12, 39/193, C12N 7/01

US CL : 424/199.1, 202.1, 218.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/199.1, 202.1, 218.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, Caplus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/37911 A1 (ORAVAX, INC.) 03 September 1998, claims 1-8.	1-6
Y	BRAY et al. Construction of Intertypic Chimeric Dengue Viruses by Substitution of Structural Protein Genes. Pro. Natl. Cad. Sci., USA. November 1991, Vol. 88, pages 10342-10346, see entire document.	1-6
Y	VENUGOPAL et al. Towards a New Generation of Flavivirus Vaccines. Vaccine. 1994, Vol. 12, No. 11, pages 966-975, see entire document.	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

16 FEBRUARY 2001

Date of mailing of the international search report

14 MAR 2001

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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Authorized officer

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703-308-0198
CENTER 1600

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/32821

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-6

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/32821

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-6, drawn to a chimeric live, infectious, attenuated virus.

Group II, claim(s) 7-12, drawn to a method of using a chimeric, live, infectious, attenuated virus.

Group III, claim(s) 13-18, drawn to a nucleic acid molecule encoding a chimeric live, infectious, attenuated virus.

Group IV, claim(s) 19-22, drawn to a method of using a yellow fever virus vector.

and it considers that the International Application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The invention of group I does not make a contribution over the prior art as evidence by Chambers et al. (WO 98/37911). Since the chimeric, live, infectious, attenuated yellow fever virus is taught in the art as evidence by Chambers et al. (WO 98/37911) the invention lacks unity on the invention as defined by PCT Rule 13.2. The cited reference proves that the technical feature of the Group I does not make a contribution over the prior art, accordingly, the unity of the invention is lacking among all groups.